Abstracts
Herein we report on the identification of a DNA molecule (aptamer) towards osteogenic progenitor cells from different origins. The aim was to use the aptamer as a capturing molecule for the immobilization of biomaterials or for specific selection of subpopulations. The aptamer was generated using the technique called SELEX (systematic evolution of ligands by exponential enrichment). In this study, we were able to generate for the first time an aptamer binding to osteogenic induced jaw periosteal cells (JPCs). Similar binding affinities were detected towards mesenchymal stem cells (MSCs) derived from placental tissue and bone marrow. In contrast, no selective binding affinity of the aptamer to chondrogenically and/or adipogenically induced MSCs or other cell lines tested could be measured. Comparative analyses of aptamer-positive and -negative sorted JPCs resulted in no significant differences concerning osteogenic marker gene expression. However, we detected stronger mineralization and a higher content of calcium phosphate precipitates of the aptamer-positive cell fraction under 2D- and 3D-culture conditions. Summarizing our results, we were able to generate an aptamer that binds with a higher affinity towards osteogenically induced JPCs. Further studies should clarify the target molecule of the identified aptamer and modification attempts should be undertaken to stabilize the DNA molecule.
Sulfated Polysaccharide Fucoidan and Mesenchymal Stem Cells Can Enhance Chondrogenesis

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Mesenchymal stem cells can be isolated from various tissues and expanded in vitro with the capacity to differentiate into a variety of cell lineages, including adipocytes, osteocytes, chondrocytes, muscles, and stromal cells. Fucoidan is a sulfated polysaccharide found mainly in various species of brown algae and brown seaweed. Polysaccharide with the sulfate groups, such as heparin reported that they impact on the differentiation of bone and cartilage. Purpose of this study, mesenchymal stem cells and fucoidan can enhance chondrogenesis. In vitro was cells with chondrogenic differentiation medium for releasing precipitated by centrifugation and cultivated for 21 days in incubator and exchanged with fresh medium every 3 days. In vivo was fucoidan added to the culture medium of zebrafish embryos 24 hours after fertilization, respectively 48, 72, and 96 hours, the extend of cartilage formation was confirmed. Total RNA was extracted mRNA of cartilage-specific marker genes was confirmed by RT-PCR, Safranin O staining and Alcian blue staining. Fucoidan induced group pellet size is larger than control group. In vitro and in vivo results were fucoidan is more effective in cartilage formation. Fucoidan was effective in the formation of cartilage. In the near future, fucoidan is effective material to replace it is considered able to cartilage regeneration or cell therapy.
Injectable hydrogels that can provide minimally invasive cell delivery for therapeutic applications represent a powerful tool in regenerative medicine. Here, the performance of proteolytically-degradable alginate (ALG) hydrogels as vehicles for human MSC was evaluated. ALG were functionalized with the matrix metalloproteinase (MMP)-sensitive peptide PVGLIG. This peptide, in free form or conjugated to ALG molecules, did not promote dendritic cell maturation in vitro, suggesting low immunogenicity. 3D cultured MSC were able to spread and form interconnected cellular networks within MMP-sensitive hydrogels (PVGLIG-RGD-ALG), but remained round within their MMP-insensitive counterpart (RGD-ALG). MSC in MMP-sensitive hydrogels also secreted higher amounts of MMP2. MSC-laden 3D matrices were embedded in a tissue mimic, in vitro, and cells were able to migrate from MMP-sensitive hydrogels and invade the surrounding matrix. In vivo, subcutaneously implanted MSC-laden MMP-sensitive hydrogels were more rapidly degraded and infiltrated by endogenous cells, new tissue and blood vessels. In all cases, softer (2wt% ALG) matrices presented more signs of degradation than stiffer ones (4wt% ALG). Finally, MSC were able to evade the hydrogels, directly interact with host cells, and synthesize their own extracellular matrix.

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Osteoblast Cell Response to a 3D Biomimetic Scaffold Grafted with SPARC

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SPARC (secreted protein, acid and rich cysteine) is a glycoprotein that exhibits counter-adhesive effects during tissue repair and differentiation and is involved in multiple coordinated functions during bone formation (initiating mineralization and promoting mineral crystal formation). [1, 2] However, its specific role is still not fully understood. This work aims at analyzing SPARC adsorption on a 3D scaffold that mimics bone extracellular matrix (ECM) based on collagen nanofibers and nanohydroxyapatite agglomerates incorporated between them. In vitro assays were carried out on these 3D matrices and its influence on MC3T3-E1 osteoblast cell line culture in terms of cell adhesion, morphology and proliferation was investigated. This innovative biocomposite immobilized with SPARC is a promising candidate for bone tissue regeneration similar to human ECM as well as in biomedical application for the treatment of bone diseases.
Neural precursor cells rest within a specific microenvironment of the mammalian dentate gyrus (DG). Upon activation, they proliferate, differentiate to granule cells and get incorporated into adult neural networks. This process of neurogenesis is highly regulated with mandatory contribution of the signaling between extracellular matrix (ECM) and corresponding integrins. In order to reassemble this unique niche with star-PEG-heparin hydrogel, this study identified key mediators of the NPC integrin-matrix interaction that may contribute to optimized NPC in vitro cultures. QPCR studies of various integrins showed up-regulation of integrin alpha 5, 6 and v as well as beta 1 and 8. Analysis of NPCs during the isolation and purification procedure unveiled an uniform adaptation for all integrins. Adhesion assays of NPCs to basal membrane derived laminin, different recombinant laminins (111, 211, 411, 511, 521), fibronectin and vitronectin showed a strong association to laminin alpha 5 isotypes but only moderate adhesion to laminin 111 and vitronectin. As shown by blocking experiments the adhesion to laminin-coated surfaces was by mediated by integrin alpha 6, whereas integrin alpha v and 5 were involved on fibronectin. Furthermore, proliferation was increased on recombinant laminin 511 and 521 surfaces. The characterization of the integrin-matrix interaction of NPCs identified integrin alpha 6 and laminin alpha 5 isotypes as potential key players for cell adhesion. Both molecules are reported to be involved in the regulation of survival. Hence, we suggest the incorporation of laminin alpha 5 or functional fragments of it into the star-PEG-heparin hydrogel system.
Salidroside Induces Angiogenic Differentiation and Protects Against Oxidative Stress in Bone Marrow Derived-Endothelial Progenitor Cells via Akt/mTOR/p70S6K and ERK1/2 Pathways

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Despite intense research efforts, the repair of large bone defects is still not satisfactory and remains a major orthopaedic challenge. Tissue-engineered bone has emerged as an effective strategy to overcome the challenge, but the vascularization of bone graft is an obstacle which restricts its performance. The aim of this study was to investigate whether Salidroside (SAL), a phenolic glycoside with potent anti-inflammatory and anti-apoptotic properties that used in Traditional Chinese Medicine, could induce cell proliferation and capillary formation and inhibit oxidative induced-apoptosis in bone marrow derived-endothelial progenitor cells (BM-EPCs) and, if so, through what mechanisms. We observed that SAL increased cell proliferation, migration, extracellular matrix (ECM)-adhesion and capillary-like structure formation in BM-EPCs in a dose-dependent manner. Furthermore, SAL enhanced the secretion of vascular endothelial growth factor and production of nitric oxide, abrogating hydrogen peroxide (H2O2)-induced cell apoptosis and reactive oxygen species formation. When examined the signaling pathways in SAL-treated BM-EPCs, we found that SAL promoted proliferation and endothelial gene expression, stimulating the phosphorylation of Akt, mammalian target of rapamycin (mTOR), and ribosomal protein S6 kinase (p70S6K), as well as ERK1/2. Additionally, SAL reversed the induced effect of H2O2 on phosphorylated SAPK/JNK and p38, and promoted the level of anti-apoptotic proteins Bcl-xL after H2O2 induction. Taken together, these observations suggest that SAL has a strong potential as a novel pro-angiogenic component supporting bone tissue vascularization and protecting against endothelial dysfunction.
Sericin Protein Obtained from Silk Cocoon Helps Chondrogenesis

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Sericin is one of the silk proteins produced by mulberry silkworm (Bombyx mori). Silk is composed of two types of protein; sericin and fibroin exist as a ratio of 25: 75. Biocompatibilities of these silk proteins have attracted attention as biomaterial. In the previous study, we found that silk fibroin and sericin were effective on chondrocyte growth and chondrogenesis. Especially, sericin was more excellent than fibroin. In this study, we prepared fraction of sericin according to molecular weight by Sephacryl S-300 and investigated the effect on chondrogenesis. All of the fractions were capable of producing cartilage –specific ECM. Also, expression levels of genes related chondrogenesis such as collagen type II, collagen type X and sox 9 were increased. These results suggest the possibility of silk sericin for cartilage regeneration.
Scaffolds Based on Calcium Phosphate Glasses Induce Bone and Blood Vessels Formation: An In vivo Study

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Nowadays, the in situ repair is the approach with the highest potential of application in tissue engineering. Previous reports pointed out the role of scaffolds containing a calcium phosphate glass (G5) in the induction of chemotaxis, angiogenic processes and osteogenic differentiation in vitro. However, in order to reach the final clinical application, in vivo studies are necessary to evaluate the ability of PLA/G5 to induce bone regeneration. The aim of this study was to evaluate the role of PLA/G5 on the regeneration of rat’s femur condyle defects.

Scaffolds containing PLA (95/5), PLA/G5 (50% G5) or empty (negative control) were applied into femur condyle defects of 2.8 mm diameter. After 3, 15, 30 and 60 days rats were sacrificed and posterior legs were retrieved. Angiogenesis and Osteogenesis PCR arrays were performed on samples obtained after 3 and 15 days. Ex vivo µCT, Goldner Trichrome staining and immunohistochemistry analysis were performed on samples after 3, 15, 30 and 60 days to reveal new bone, blood vessels and the presence of CaSR.

Results from PCR arrays revealed that PLA/G5 induces upregulation on 40% and 96% of the genes involved in angiogenesis and osteogenesis at day 3 respectively. After 15d PLA/G5 promoted the expression of proangiogenic signals on 40% of genes. qPCR demonstrated that PLA/G5 increased 4 times VEGF expression compared with controls. Analogously, osteogenic arrays showed few differences between PLA and PLA/G5. However qPCR revealed 4 times upregulation of BMP2 on PLA/G5 compared with PLA. µCT demonstrated that PLA/G5 at 30d and 60d promoted the higher BV/TV ratio. Histology results are currently under analysis. Results pointed out a higher potential of PLA/G5 to induce angiogenesis and bone formation in vivo.
Combination of a New Injectable Multiphasic Bone Substitutes Based on Gel-Coated OSPROLIFE HA/TTCP Granules with Bone Marrow Concentrate: Towards a Single-Step Procedure

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The next-generation of bone graft substitutes aims at improving their handling properties and their clinical efficacy. Currently, injectable calcium phosphates bioceramics are combined with mesenchymal stromal cells (MSC) for effective bone regeneration. MSC are contained in bone marrow (BM); many devices have been developed to isolate the MSC in the nucleated cell (NC) fraction of bone marrow concentrate (BMC). The purpose of this study was to examine the in vitro compatibility of a new injectable multiphasic bone substitutes (MBS) based on gel-coated OSPROLIFE HA/TTCP granules (Eurocoating S.p.A) with BMC. BMC was obtained using the IOR-G1 device (Novagenit S.r.l). The concentration and viability of NC were evaluated in the BM and after isolation and cell recovery was calculated. Gel-coated granules loaded in syringe were combined with BMC diluted in gel cross-linking solution, then the product was maintained in culture for in vitro compatibility test. Cell viability was assessed at time 0, 24, and 96 h using LIVE/DEAD staining kit. The distribution of calcein stained cells in the product was studied through confocal microscopy. NC concentration in the BMC increased by a factor of 7.4 in the BMC compared to BM. Compatibility analysis highlighted that cells remained viable hereafter the combination with the gel-coated granules and viability was maintained. Confocal imaging showed the cells suspended in the gel after a 6-day culture. This fact confirms the protective role of gel in maintaining cells viability. Injectable MBS based on gel-coated OSPROLIFE HA/TTCP granules were proved to be compatible and their easy combination with BMC can lead to a more clinically successful “single-step procedure” for bone regeneration.

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Effects of Platelet-Rich Plasma on Cartilage Regeneration in Chondrocyte-Encapsulated Gelatin Hydrogels

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This study presents the effect of platelet-rich plasma (PRP) incorporated into gelatin hydrogels on cartilage regeneration. PRP is rich in growth factors and cytokines that can stimulate tissue regeneration. Enzymatically cross-linked gelatin hydrogels served as an injectable carrier for PRP and cells. For in vitro 3D culture of chondrocytes, cells were suspended in aqueous solutions of gelatin-poly(ethylene glycol)-tyramine with and without PRP, and then subjected to enzymatic cross-linking. PRP contained hydrogels showed enhanced cell viability after 7 days of culture. RT-PCR results showed that the expression level of Sox-9 markedly increased when cells were cultured in the PRP contained hydrogels for 3 days. In addition, the expression level of type II collagen evidently increased in the hydrogels at day 7. Interestingly, CB1 that prevents cartilage matrix degradation gradually increased in the PRP-contained hydrogel, implying controlled release of growth factors in PRP. In vivo studies demonstrated that, though no remarkable changes were observed, the PRP contained hydrogel had a relatively thick connective tissue and revealed early formation of chondroid matrix with rimming spindle cells. Therefore, PRP incorporation into the gelatin hydrogel can be used to induce proliferation and differentiation of chondrocytes effectively.
Tissue Engineering as a Useful Tool in a Bioprospecting Program of a Developing Country

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Tissue engineering uses cells, engineering and materials methods, as well as physiochemical factors to improve or replace biological functions, leading to great advances in regenerative medicine. Nevertheless, tissue engineering products, like artificially-created tissues have allowed other advances in biomedical sciences, for example, understanding the laying mechanisms in cell physiology and the effect of chemical molecules. In this matter, at the Tissue Engineering Laboratory of the Costa Rica Institute of Technology, we have several years of experience culturing skin cells to treat extensive burns, ulcers and genetic diseases, and more recently, to learn more about skin regeneration and its cellular processes. Considering the great biodiversity available in Costa Rica’s territory (5% of the whole world biodiversity), we proposed a strategy to evaluate the effect of 3 vegetal species extracts (Rubus adenotrichus, Phyllanthus niruri and Jatropha curcas), traditionally known for their anti-carcinogenic activity and grown in vitro by colleagues in our Institution, with findings at the moment that Rubus sp. actually protects skin cells against DNA damage induced by UVB and influences cellular differentiation.

We consider this kind of research as of great benefits for our country, since it allows the specialization of new professionals, make sustainable use of natural resources, and the possibility of finding new therapeutic substances.
Biological Activity of a Standardized Freeze-Dried Platelet Derivative to Be Used as Cell Culture Medium Supplement

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Serum of animal origin and in particular fetal bovine serum are the most commonly utilized cell culture medium additive for in vitro cell expansion. However, several scientific-technical/ethic concerns exist with regard to the use of these animal sera. Among the possible alternatives to the animal serum, platelet derived compounds have been proposed since more than ten years. In this study we describe the preparation of a standardized platelet rich plasma (PRP) derivative obtained starting from human certified buffy coats samples with a defined platelet concentration. We tested the biological activity of the product after freeze-drying and gamma-irradiation and we compared its use as culture medium additive, against the “traditional” bovine serum (FCS 10%), to sustain human bone marrow Mesenchymal Stem Cells (MSC) clone formation (CFU-f) and proliferation. Further, the PRP effects on cell proliferation were evaluated also on cell line cultures. We observed that the PRP addition to the culture medium increased the MSC colony number and average size. In primary cell cultures and in cell line cultures, the PRP promoted cell proliferation also in conditions where the FCS had not a proliferation stimulating effect due to either the nature of the cells and the tissue of origin (such as human articular chondrocyte from elderly patients) or to the critical low density cell seeding (such as for Hela cells). In summary the standardized PRP formulation would provide an “off the shelf” product to be used for the selection and expansion of several cell types also in critical cell culture conditions.
The Effects of Estradiol on Rabbit Articular Chondrocytes in 3D Culture

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The aim of this study was to investigate the biological effects of estradiol on the chondrocytes in 3D bioreactor culture. We performed the chondrocytes culture in 3D bioreactor with 10⁻⁶ and 10⁻⁸ M estradiol of female chondrocytes. MTT assay, proteglycan synthesis, and quantitative real time-PCR were evaluated on the 0, 1, 3 and 7 day to investigate the viability and dedifferentiation of chondrocytes. The result revealed increase MTT assay in the group of 10⁻⁶ M estradiol on 7 day compared to other groups. All the expression of Col-II mRNA, aggrecan mRNA, and TIMP-3 mRNA increased significantly following incubation with 10⁻⁶ M β-estradiol at 1, 3, and 7 day. Furthermore, the expression of MMP-3 mRNA was suppressed in the group of 10⁻⁶ M than the control group and 10⁻⁸ M with significant difference. Immunocytochemical staining was performed at 0, 3, and 7 day. The result suggested greater collagen II expression in the chondrocytes incubated with 10⁻⁶ M β-estradiol than other groups. The positive influence on articular cartilage metabolism of condrocytes incubated with 10⁻⁶ M estradiol observed in 3D culture. As present results of our study, sex hormones provided positive biological effect on articular chondrocytes, especially with estradiol. Furthermore, we expected further in vivo animal studies to test the effect of 10⁻⁶ or 10⁻⁷ M estradiol in chondrocytes.
Ongoing efforts to search for bioactive substances for bone diseases have led to the discovery of natural products with substantial bioactive properties. In this present study, an osteoblast activating-peptide was isolated from biodiesel by-products of microalgae, *Nannochloropsis oculata*. To utilize biodiesel by-products of *N. oculata* and evaluate their beneficial effects, enzymatic hydrolysis was carried out using commercial enzymes such as alcalase, flavourzyme, neutrase, PTN, and protamex, and alcalase hydrolysate exhibited the highest osteoblastic differentiation activity. Using consecutive purification by liquid chromatographic techniques, an osteoblast-differentiatory peptide was purified and identified to be a peptide (MPDW, 529.2 Da) by the tandem MS analysis. The results showed that purified peptide promotes osteoblast differentiation by increasing expression of several osteoblast phenotype markers such as alkaline phosphatase (ALP), osteocalcin, collagen type I, BMP-2, BMP2/4 and bone mineralization in both human osteoblastic cell (MG-63) and murine mesenchymal stem cell (D1). In addition, the purified peptide induced phosphorylation of MAPK and Smad pathway in both cells. These results suggest that peptide possesses positive effects on osteoblast differentiation and may provide possibility for treating bone diseases.
Determinant of Salinomycin Effective Dose for Glioblastoma Cells

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Salinomycin (SAL), which is a polyether antibiotic commonly used as an anticoccidial drug, has recently been shown to have antitumor properties (1,2). In order to investigate the effective dose of SAL for brain tumor treatment, it is aimed to determine cellular activities of T98G human glioblastoma cell line within several concentrations of SAL exposure.

T98G cells were seeded on 24 well tissue culture plates with a cell density of 2x10⁴ cells/well. After cell adhesion, supernatant was removed and refreshed with different concentration of SAL (0, 0.1, 0.5, 1, 5 and 10 μM). The mitochondrial activity of the cells was determined using the MTT assay, cell viability was identified with toxicology test (TOX 6 kit, Sigma) and the results are presented as mean IC⁵₀ values after 24, 48, 72 and 96 hours of the culture period. Cellular morphology was observed upon fluorescence microscope.

According to the toxicology and MTT tests, a significant decrease on cell viability and cellular activity was determined for all SAL doses after 24 h, which is confirmed by IC⁵₀ values. Moreover, the lowest cell viability and cellular activity ratios were determined for 5 and 10 μM SAL and after 96 h incubation the most effective dose was found to be 10 μM SAL. Furthermore, microscope images of T98G cells showed significant cell membrane and F-actin deformations with 10 μM SAL dose exposure.

In conclusion, our results showed that for brain tumor therapy salinomycin can be a potent cancer drug with the effective doses of 5 and 10 μM SAL concentrations.
The problem of environmental pollution caused by different hazards has a special importance for Kazakhstan. The accumulation of xenobiotics in the environmental components (pesticides, heavy metals, hydrocarbons, rocket fuel components, etc.) leads to significant biochemical, genetic and morpho-physiological changes in the organism. The problem of finding methods of protection of the organism from pollutants along with activation of natural protective systems has the utmost importance. One of the promising sources of growth factors, which can enhance the immune response of the plants, is cyanobacteria’s (blue-green algae). In the process of life, they accumulate and secrete the metabolites with high biological activity into the environment. Therefore, this study aimed to investigate the properties of protective bioactive substances produced by various associates of microalgae. The metabolites with high biological activity from different associate algal species were tested as modifiers of chemical mutagenesis. Such events as chromosome aberrations were studies. *Hordeum vulgare L.* were used in experiments. The seeds of barley were exposed to the cadmium salts and metabolites from cyanobacteria. The results of the cytogenetic analyses on plant test-objects revealed that bioactive compounds made from *Cyanoprocaryota*, *Bacillariophyta* and *Chlorophyta* algae associates did not show mutagenic activity. The combination of cadmium salts with bioactive compounds resulted in the modification of cytogenetic effects caused by cadmium sulfate and chloride. Additional exposure to bioactive compounds led to a significant reduction of the structural mutations induced by cadmium. The obtained results indicate about antimutagen potential of metabolites of tested microalgae associates.
Breast cancer is one of the major causes of death among women. Due to the anticarcinogenic effect of resveratrol is quite a broad spectrum of employees in this area has been the focus of attention. In the present study, we aimed to investigate the in vitro effects of resveratrol (RSVL) on breast cancer cell line MCF7 cancer cells with various techniques. For this purpose, MCF7 (1x10⁵) were inoculated in 6-well plates. All cell lines were maintained in DMEM, supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 U/l penicillin and 100 U/l streptomycin and were cultured at 37°C in a humid atmosphere consisting of 5% CO₂ and 95% air. After 2 hours later, RSVL was used at various concentrations (0,1,10,20,40μM) for 4 days. We determined the proliferation capacity and viability using WST, and 72 hours later apoptosis level using AnnexinV-PI staining by using flow cytometer. As a result, we found a sharp decline in proliferation 40μM concentration (1,27 ± 0,01) compared to control group (2,15 ± 0,01) (p<0.001). Application of RSVL resulted in a dose-dependent decrease in WST-1 proliferation assay (1,10,20 μM) (1,82 ± 0,01 ; 1,62 ± 0,01 ; 1,55 ± 0,01). We obtained sphere using the same cell lines. In order to determine the effect of different doses of RSVL on this sphere ATP tumorchemosensitivity test was performed. We obtained similar results with AnnexinV-PI staining according to the percentage of apoptotic cells (dose 1,10,20,40 μM) (21,08 ± 0,05 ; 36,68 ± 0,09 ; 82,64 ± 0,13 ; 91,84 ± 0,08) compared to control (16,72 ± 0,11) (p<0.001).

In conclusion, we are in the belief that resveratrol providing a major direct support to stem cell therapy, as an alternative treatment option in the treatment of many diseases, is a big step to be taken noted promising results.
Auricular Cartilage Novel Protocol to Coat Covalently Polymeric Surfaces with Bioactive Organic Glasses for Tissue Engineering

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Up to now, hybrid scaffolds developed for tissue engineering applications have generally been fabricated by a simple dispersion of an inorganic component into a polymeric matrix. As a result, the inorganic domain (i.e the bioactive phase) is often masked by the organic one and unperceived by cells. The aim of this study is to develop a new strategy for the production of materials whose bioactive compound is totally exposed on its surface, in order to improve cell-material interactions. Using the sol-gel method and several surface treatments (hydrolysis, activation, coupling agent functionalization), polylactic acid electrospun fibers have been coated with two bioactive glass compositions successfully (Si-Ca-P2 system). This protocol allows the fabrication of scaffolds with good hydrophilic features, enhanced mechanical properties, a great cellular adhesion, and tailored nanostructured topographies and stiffness by just changing glass composition. In addition, the obtained scaffolds were very flexible due to the use of an organic glass rather than a stiff inorganic one. Concerning bioactivity, cells could immediately detect the glass exposed at the fibers surface, as well as the bioactive ions released during glass degradation. That may allow them to differentiate towards a specific cell fate depending on these chemical cues. As surface properties influence cell functionalities (proper composition and appropriate structure for example), a broad range of biomedical applications can be targeted by modifying the coating characteristics. This novel coating protocol constitutes therefore a significant improvement in the design and surface engineering of biomaterials.
A Peptide Isolated from Fermented Microalgae, *Pavlova Lutheri* (Haptophyceae), Induces Human Osteoblastic Differentiation through ALP and MAPK Activation

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A osteoblast-differentiative peptide, EPQWFL (MW = 908.9 Da) was purified from the fermented microalgae (*Pavlova lutheri*), which is product of yeast fermentation by *Hansenula polymorpha*. Our results indicate that the isolated peptide from fermented *Pavlova lutheri* (PFPL) can increase activity of alkaline phosphatase (ALP) as a phenotypic marker for early-stage osteoblastic differentiation. Furthermore, the results showed positive effects of peptide on ALP and bone morphogenic protein-2 (BMP-2) as important factors for bone formation and mineralization. To elucidate the mechanisms by which the peptide acted, we examined its effects on 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced MAPKs activation and determined that the peptide treatment significantly reduced MAP (p-p38) kinase in MG-63 cells. The present study may provide new insights in the osteoblastic differentiation of purified peptide and possibility for its application in bone health supplement.
Bioactive Self-Assembled Peptide Hydrogels For \textit{In Vivo} Bone Tissue Regeneration

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Biomineralization is deposition of calcium by cells to their extracellular matrix (ECM), and it is an essential mechanism of bone and teeth formation in humans. Biomineralization is especially important in adults for tissue regeneration in bone defects. Both collagenous and non-collagenous ECM molecules regulate cellular behavior such as adhesion, migration, proliferation and differentiation during biomineralization process. Glycosaminoglycans (GAGs), one of the non-collagenous components of ECM, have important functions in bone remodeling by stabilizing growth factors and enhancing growth factor-receptor interactions, thus, affecting cellular proliferation and differentiation. In this study, effects of GAG mimetic peptide nanofibers on mechanisms of biomineralization were investigated in a bone regeneration model \textit{in vivo}. GAG mimetic peptide nanofiber hydrogel was used to induce biomineralization in damaged bone tissue. GAG mimetic peptide nanofibers triggered healing of the bone defect, which was observed by formation of new collagen fibers and increase in newly formed bone volume fraction investigated by histology and Micro-Computed Tomography studies, respectively. Expression of bone-specific proteins such as osteocalcin and osteopontin was also observed by immunohistochemistry staining. These results show that GAG mimetic peptide nanofiber system can be used as an efficient therapeutic agent for the treatment of bone defects.
Effects of Biosilicate Scaffold and Low Level Laser Therapy on one Consolidation in Rats

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Fractures of delayed consolidation and fractures with non-union are commonly found in medical practice and are associated with high morbidity and mortality. Within this context, biochemical and biophysical resources have been studied in an attempt enhance bone healing. Among these may be highlighted the use of bioactive materials and low level laser therapy (LLLT), which seem to induce osteogenesis and stimulate fracture healing. The aim of this study was to investigate the effects of LLLT and a novel bioactive material (Biosilicate) used associated or not, on consolidation of induced tibial bone defects in the rats. Forty male Wistar rats (± 300 g) were randomly divided into four groups, with 10 animals each: group control bone defect without any treatment (CG); group bone defect irradiated with LLLT (LG); group bone defect treated with implantation of scaffolds Biosilicate (BG); group bone defect treated with implantation of scaffolds Biosilicate and LLLT (LBG). The animals were submitted to laser irradiation (830 nm, 100 mW, 120 J/cm², energy of 3.4 J, with a irradiation time of 34 s) at a single point on the bone defect for eight sessions, on alternate days. Morphological analysis revealed that the LG showed a higher amount of newly formed bone and better tissue organization in relation to other groups. The immunohistochemistry analysis showed that the BG and LBG produced a higher expression of COX-2 (cyclooxygenase-2) and RUNX-2 (runt-related transcription factor-2) in the circumjacent cells of the biomaterial. Our results indicate that both treatments had pro-inflammatory effect and osteogenic potential 15 days after surgery, but the LLLT was more effective in bone repair when compared to the biomaterial or the two resources associated.

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Ongoing efforts to search for bioactive substances for bone diseases have led to the discovery of natural products with substantial bioactive properties. In this present study, the seventeen substances from marine bio-resources evaluated osteobastic differentiation. The marine resource including three kind fucoidan from *Ecklonia Cava*, *Hizikia fusiforme* and *Undaria Pinnatifida*, chitosan oligosaccharide (Mixture, >10 kDa, 10-5 kDa, 5-3 kDa, 3-1 kDa, <1 kDa), Ca-citrate and protein from abalone (*Haliotis discus hannai*) shell, muscle powder and hydrosates from abalone (*Haliotis discus hannai*), phlorotannin from *Ecklonia Cava*, fish collagen, hoki (*Johnius belengerii*) bone extracts and casein phosphopeptide (CPP). The results showed that two fucoidan (*Ecklonia Cava* and *Undaria Pinnatifida*), phlorotannin, and one chitosan oligosaccharide (>10 kDa) promotes osteoblast differentiation by increasing expression of several osteoblast phenotype markers such as alkaline phosphatase (ALP), osteocalcin, osteopontin and bone mineralization in human osteoblast like cell line (MG-63) compared other substances. These results suggest that four substances from marine resource possesses positive effects on osteoblast differentiation and may provide possibility for treating bone diseases.
Establish An Alternative Method for Assessing Remnant Gal-antigen Induced Immunotoxicity*: Part 1: α-1,3 GT+/− Recombinant C57BL/6 ES Cell Cloning

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Animal tissue derived biological materials are widely used in tissue engineering and regenerative medicine. However, the immunological problem arose from different species between human and animal is a big issue. Unlike most mammals, humans do not express α-1,3-galactosyl (α-Gal) due to that humans lack a functional α-1,3-galactosyltransferase (α-1,3GT) gene, and produce abundant anti-Gal antibodies. Anti-Gal antibodies are the primary effectors of human hyper-acute rejection (HAR), as well as chronic immunological responses, against animal tissue-derived biological materials. However, traditional immunotoxicity assessment by using animals is not able to evaluate the gal-antigen induced immunotoxicity. To establish an alternative method for assessing the gal-antigen induced immunotoxicity, we develop an α-1,3 GT gene knockout (α-1,3 GT−/−) mouse. Here, it is presented that the construction of α-1,3 GT gene knockout vector by using bacterial artificial chromosome (BAC) homologous recombinant technique and cloning of α-1,3 GT+/− C57BL/6 ES cell. Because the majority of the coding region of the α-1,3 GT gene, including the sequences encoding the catalytic domain, is located in exon 9. Deleting of exon 9 will result in functional inactivation of the gene. So, exon 9 is replaced by inserting a NeoR-pA fragment with a homogenous arm located in up- and down-stream of exon 9 (pBCTG-A-NeoR-B), and by saving it to pDTA-GT-C vector a homologous recombinant vector pDTA-GT-A-B-C was successfully constructed. By injecting pDTA-GT-A-B-C to the C57BL/6 ES cells, 200 of G418 selected clone were obtained. These results will be used for the next step to generate α-1,3 GT−/− mouse.

Key words: α-1,3-galactosyl (α-Gal); α-1,3-galactosyltransferase (α-1,3 GT); gene knockout mouse; homologous recombinant technique; immunotoxicity; assessment.
Novel Osteochondral 3D Model as an In Vitro Technological Platform for In Vitro Screening

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Adequate in vitro models to evaluate the performance of relevant tissue engineered constructs are still a major demand of the field. Apart from minimizing animal experimentation, in vitro models feature the possibility of addressing species divergences regarding tissues regeneration pathways, and provide reproducible platforms avoiding the heterogeneity of the in vivo models. The aim of this project is to create an osteochondral tissue in vitro model. As proof-of-concept, a bilayered sponge-like scaffold was developed to act as a template for co-culturing rabbit adipose stem cells (rASCs)-derived osteoblasts and chondrocytes. Bilayered low acyl gellan gum (LAGG)-LAGG/hydroxyapatite (HAp) sponge structures were produced respectively integrating cartilage- and bone-like layers and creating a gradient in composition. The freeze-dried bilayered scaffolds composed by LAGG2%(w/v)/LAGG2%-HAp20% and 30%(w/w) have two integrated layers, with a gradient of HAp in the bone-like layer that, unlike cartilage-like layer, present a bioactive behavior. The bilayered structures possess about 90% porosity, 500 µm of pore size and 85% interconnectivity as determined by micro-computed tomography analysis. Swelling and degradation tests revealed that the structures can absorb about 120% of their weight in water and lost 10% of their mass after 30 days in phosphate buffered saline. In vitro studies with rASCs from Fat Pad are being performed to study cell adhesion, proliferation and differentiation. The results have shown that the developed bilayered scaffolds have a great potential for finding application as a screening platform of new therapeutic approaches for the treatment of osteochondral tissue disorders.
Myocardial infarction (MI) is characterized by functional deterioration of the myocardium downstream a coronary occlusion. When untreated, MI leads to the formation of a non-functional scar to replace the damaged tissue and thus result in congestive heart failure. The pre-clinical evaluation of newly developed therapies for MI is conducted in experimental models of MI, e.g. by permanent ligation of a main branch of the left coronary artery. Infarct size, defined as the percentage of the left ventricle affected by coronary artery occlusion, is a fundamental measure for the assessment of the morphological and functional consequences of infarction.

The measurement of the infarct size is traditionally performed by manually delineating the infarcted and normal tissue areas in cross sections of the left ventricle. However, this is a time-consuming, arduous and prone to bias process.

The aim of this work is to standardize and simplify infarct size calculation in rodent models of MI. Hence, we developed a software application for the automated quantification of infarct size – aMIquant - based on an adaptable anatomical model (Fig.1). From the adapted model we obtain a good estimation of the histology of the heart section under analysis. Experimental validation, performed by comparing aMIQuant results with manual annotation, demonstrates a strong measurement correlation with discrepancies below 11%.

Plasmonic Scaffolds for Deliberate Regulation of Transgene Expression

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Recent efforts have led to the development of systems capable of precisely delivering thermal energy and heat-inducible transgenes. Optical methods exploit the properties of plasmonic nanoparticles to absorb photonic energy from near-infrared (NIR) laser light and convert it into heat which activates gene expression driven by heat shock protein (HSP) promoters. NIR light penetrates deep into human tissues, which are relatively transparent to light within the “water window” at 650-1100 nm. These properties make NIR irradiation of plasmonic nanomaterials a reliable source of hyperthermia for in vivo activation of transgenes linked to HSP promoters, with excellent spatial definition. To activate therapeutic genes only at the time and for the duration required for effective therapy and to avoid inadvertent activation of hsp promoter-controlled transgenes in non-target tissues, we built regulatory circuits that combine the promoter of the human hsp70B gene and a small molecule-activated gene switch. In this work, hollow gold nanoparticles were conjugated to biocompatible three-dimensional scaffolds, fibrin hydrogels that polymerize in situ. Human cells harboring a fluorescent reporter gene controlled by a heat-activated and rapamycin-independent gene switch were encapsulated in scaffolds containing those nanoparticles. Viability of cells encapsulated within the hydrogel was not affected by the plasmonic structures, indicating their good biocompatibility. Transgene expression was activated by NIR irradiation in the presence of rapamycin, indicating that this approach may provide unique capabilities for tissue engineering protocols where spatial and temporal bioavailability of growth factors plays a fundamental role.
Quantitative Phenotypic Markers to Monitor Liver Regeneration Failure

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Excessive accumulation of extracellular matrix results in fibrosis which is the hallmark in chronic liver diseases. Fibrotic progression is closely related to regeneration failure and neoplastic generation, so that monitoring the histo-pathological development of liver fibrosis is important for accurate diagnostic and treatment efficacy evaluation with drugs or tissue-engineered constructs. The conventional method, histological staining of biopsy samples followed by semi-quantitative scoring, has limited sensitivity and operator-dependent variations. We applied nonlinear optics microscopy, such as two-photon excitation fluorescence (TPEF), second-harmonic generation (SHG) and coherent anti-Stokes Raman scattering (CARS), for the quantitative imaging of liver fibrosis progression. To strengthen the application of the potential 3D imaging of the whole liver organ using nonlinear optics microscopy, we developed a multi-foci multi-photon microscope (MMM) which significantly increases the imaging speed by 64 times of the conventional nonlinear microscope. We further demonstrated the potential application of nonlinear endomicroscopy for fibrosis assessment by liver surface scanning using an intra-vital non-linear microscope together with laser-speckle imaging modality. The image analysis methods were developed to characterize tissue morphological, structural and functional states to recapitulate the tissue-level architectural changes as what pathologists see. Thus, these techniques have emerged as potential important approaches to improving our understanding of liver regeneration or failure to regeneration with an emphasis on the development of effective pharmaceutical intervention.
Contrast-Enhanced Nanofocus Computed Tomography for Virtual 3D Histopathology and Morphometric Analysis of Multiple Skeletal Tissues

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Bone tissue engineering (TE) typically involves a 3D scaffold with cells, soluble signaling molecules or their combination (i.e. TE construct) to support osteogenic processes. Like in organs, tissues formed in a TE construct have a spatial heterogeneity. Thus, measurements made in 2D, such as histological sectioning, only partially reveal the degree of change induced during tissue formation. To provide 3D quantitative information on the dynamic biological processes during in vivo tissue formation in TE constructs, we propose the use of contrast-enhanced nanofocus computed tomography (CE-nanoCT) for 3D multi-tissue imaging of cartilage and bone. To show the potency of CE-nanoCT to simultaneously assess bone and cartilaginous tissue properties in 3D, we applied it on a mouse model for osteoarthritis. We showed the equivalence of CE-nanoCT images for the scoring of the structure, quality and pathophysiology of the chondro-osseous junction by direct comparison with standard histopathology. Additionally, by using an empirical model, it was shown that a limited set of 3D cartilage characteristics that can be measured by CE-nanoCT image analysis, i.e. average and maximum of the non-calcified cartilage thickness distribution and loss in glycosaminoglycans, is predictive for the cartilage function and structure as evaluated by histopathological scoring, while eliminating user-bias.

We have shown that CE-nanoCT is a tool that allows virtual 3D histopathology as well as 3D quantification of multi-tissue systems, such as the chondro-osseous junction, and hence it will be a valid tool to obtain better an insight into the biological processes of cartilage and bone tissue formation in TE constructs.
The application of mesenchymal stem cells (MSCs) in treating rheumatoid arthritis (RA) has been made possible by the immunosuppressive, differentiation and homing abilities of these cells. A non-invasive means of assessing cell integration, tissue regeneration and cellular bio-distribution is fundamental in evaluating the success of this therapy. Previous studies have focused on the development of a standardised SPION (superparamagnetic iron oxide nanoparticle) & MRI (Magnetic resonance imaging) based protocol to image and track stem cells in vivo. In this study the suitability of this protocol was assessed using an antigen induced murine model of RA. Murine mesenchymal stem cells (mMSCS) were isolated, expanded and labelled with SiMAG (1μm). In vivo and in vitro MRI detection thresholds were determined. Cell viability, proliferation and differentiation capabilities were not affected by the proposed protocol. Upon RA induction, SiMAG labelled and unlabelled mMSCS were intra-articularly injected and joint swelling monitored as an indication of RA development over a 7 day period. A significant decrease in joint swelling was measured in both SiMAG labelled and unlabelled groups. This implies that the presence of SiMAG does not affect the immunomodulating properties of the cells. In vivo MRI scans demonstrated good contrast and the identification of SiMAG- labelled populations within the synovial joint up to 7 days post implantation. This was further validated by fluorescent microscopy. The development of a non-invasive means of imaging and tracking cells has great implications in the clinical translation of a wide range of stem cell based therapies.
Creating and working with three dimensional (3D), biologically relevant central nervous system (CNS) models present major challenges, such as selecting a suitable 3D scaffold and developing fast 3D imaging systems. Laboratory engineered hydrogel scaffolds have many advantages over alternatives, since hydrogels are typically transparent and can also mimic the properties of the natural extra cellular matrix around cells, thus providing the cells with the appropriate mechanical and biological cues. Hydrogels could thus enable the growth of neural cells and the structural and functional imaging of the artificial neuronal networks created. In this study we introduce and present the fundamental characteristics of a dense 3D neuronal network composed of rat primary cortical cells embedded in a hydrogel scaffold, and the 3D optical probing of the network using optogenetic probes. The network was transfected with viral agents for the calcium indicator GCaMP3 and for the light-gated channel Channelrhodopsin 2, facilitating the probing of a large population of neurons. We monitored the activity of large neuronal populations in our 3D network, using a fast custom-developed, temporal-focusing based imaging system with frame rates of up to 200 Hz. We will present the basic characteristics of viability and immunohistological analysis of the forming networks, and of their activity patterns. Finally, we will discuss potential applications in regenerative medicine and drug screening.
Fluorescent Markers as a Tool for Monitoring 3D Scaffold Degradation and Cellular Effect

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Current *in vivo* investigation of biomaterial implantation leads to sacrifice of excessive numbers of animals. A tool kit to monitor the degradation of materials relative to cellular activity, without the need to sacrifice animals is currently being developed for *in vivo*, real time applications. Chitosan was tagged with TRITC. Porous 3D fluorescent scaffolds were created by freeze drying. The fluorescence intensity released in the culture media during enzymatic degradation, was analysed; the scaffold structure was monitored by microCT; confocal microscopy was used to determine the fluorescence intensity in the remaining 3D scaffold. A specific fluorescence reporter gene was transfected to bone cells and cultured in the fluorescent scaffolds. MicroCT of the 3D fluorescent scaffolds demonstrated that the scaffolds were homogenous and highly porous. When immersed in enzyme media, fluorescence release was detected and corresponded to a reduction in scaffold fluorescence intensity and weight loss. Scaffolds were biocompatible and cell viability was high for prolonged culture. Cells in the scaffolds appeared to affect the degradation rate. A tool for monitoring the state of 3D scaffold degradation has been developed and verified *in vitro*. Correlations between weight loss and fluorescence intensity were observed. Fluorescent biomaterial scaffolds can enable monitoring of degradation *in vivo* over time, reporting the degradation kinetics and cell/tissue growth in real-time.
Imaging Flow Cytometry (IFC) combines microscopy image analysis with flow cytometry enabling a qualitative and quantitative study of diverse cell processes. In this study, we compared the potential of IFC to common techniques employed in membrane receptor analysis, as a tool to characterize the behavior of a fluorescent NPY Y1 receptor (Y1R) antagonist -BIBP3226*- within the mice bone marrow, and evaluate its potential for the future development of a local drug delivery system for bone regeneration. Confocal microscopy analysis, being limited by the image field selected and falling short in encompassing the whole cell population, lead to erroneous conclusions namely about the binding and internalization kinetics of the receptor, mainly due to the fact that not all bone marrow cell types express the receptor and therefore bind the antagonist. As these are very fast processes, the focusing and analysis time needed impaired the global analysis of the diverse cell population of the bone marrow, especially in live cell conditions as required by the antagonist. In the radioactivity assays, besides the safety issues raised, we were very limited by the high Kd of the radioligand that made difficult the establishment of the optimal conditions that would allow the radiolabeled-antagonist to be kept bound to the receptor without contamination from the excess radioligand on the supernatant. Overall, IFC proved to be an excellent tool for studying Y1R-BIBP3226* binding, allowing a fast, safe, qualitative and quantitative analysis of the underlying cellular processes in a diverse cell population such as the live bone marrow cells.
Dual MR and CT Imaging of Contrast Agent-enhanced Calcium Phosphate Cements

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Calcium phosphate cements (CPCs) are commonly used bone substitutes which closely resemble the mineral phase of the bone. Current imaging performed by X-ray computed tomography (CT), is restricted due to the similar radiopacity of bone and calciumphosphate-based materials. Magnetic resonance imaging (MRI) offers new high-resolution bone imaging possibilities, still is hampered by the low water content and thus short T2-relaxation time of bone and CPC. A MRI Zero Echo Time (ZTE) sequence, performing both excitation and acquisition in presence of a constant gradient, is proposed here as alternative. We especially focused on CT opacifiers like colloidal gold, and MR agents like gadolinium and Ultra Small Iron Oxide Particles (USPIO). Agents were first incorporated in a silica-based composite bead to reduce unspecific reactivity in the setting and mechanical properties of the CPC. After in vitro characterization, the composite was tested in a femoral condyle defect rat model monitored up to 8 weeks post surgery. In vivo results underlined the clear verification of proper implant placing characterized by a short-term CT contrast enhancement and a long-term MR effect. The mechanical properties of the cement were not significantly affected by the inclusion of the beads and no signs of adverse tissue reactions were noticed. In conclusion, the results supported the combination of optimized imaging modalities, such as ZTE-MRI, with smart contrast formulations.
In this study, we investigated «recipient–tumor–fluorescent stem cells » system using the methods of in vivo imaging and laser scanning microscopy (LSM). Recently, great deal of interest is investigation the function of the stem cells (SC) in tumors. We used adipose-derived adult stem (ADAS) cells of human liposome transfected with the gene of fluorescent protein Turbo FP635. ADAS cells were administrated into nude mice with transplanted tumor HeLa Kyoto (human cervical carcinoma) at different stages of tumor growth (0–8 days) intravenously or into tumor. In vivo imaging was performed on the experimental setup for epi–luminescence bioimaging (IAP RAS, Nizhny Novgorod). The results of the imaging showed localization of fluorophore tagged stem cells in the spleen on day 5–9 after injection. The sensitivity of the technique may be improved by spectral separation autofluorescence and fluorescence of stem cells. We compared the results of in vivo imaging and confocal laser scanning microscopy (LSM 510 META, Carl Zeiss, Germany). Internal organs of the animals and tumor tissue were investigated. It was shown that with i.v. injection of ADAS, bright fluorescent structures with spectral characteristics corresponding to TurboFP635 protein are locally accumulated in the marrow, lungs and tumors of animals.

These findings indicate that ADAS cells integrate in the animal body with transplanted tumor and can be identified by fluorescence bioimaging techniques in vivo and ex vivo.
Auricular Cartilage Dual Imaging of Hydrogels Formed by Hydrazone Cross-linking of Hyaluronic Acid Hybrid Nanogels

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Degradability of hydrogel biomaterials delivering growth factors (GFs) for tissue regeneration is an important factor that determines the GFs therapeutic concentration over time as well as the response of an organism to the implanted material. In current tissue regeneration applications there is a lack of knowledge about how long a material can last \textit{in vivo} at different sites of implantation as well as where exactly the degradation products are utilized \textit{in vivo}. To address these problems, we have prepared a hydrogel material enabling tracking of its resorption using fluorescence and magnetic resonance (MR) dial imaging. Hyaluronic acid (HA), the main glycosaminoglycan component of extracellular matrix, has been functionalized with hydrazide and thiol chemoselective groups. Hydrophobic pyrene ligands were attached to the hydrophobic HA backbone via thiol-ene coupling reaction. The obtained pyrene-grafted HA self-assembled in aqueous medium with the formation of hydrophobic core-hydrophilic shell nanogel particles of 400 nm size. Oleic acid-capped iron oxide (10 nm) nanoparticles were hydrophobically entrapped in the cores of the core-shell HA nanogels during the self-assembly. The nanogels were in situ transformed into a hydrogel material using orthogonal hydrazone cross-linking reaction between the remaining hydrazide groups of the nanogels and aldehyde groups of the aldehyde-derivatized HA macromolecules. Both HA nanogels and the resulting hydrogels were imaged by MRI. Degradation of the iron oxide-hybridized HA hydrogels in the presence of different amounts of hyaluronidase was studied in vitro by UV spectroscopy and MRI.
Auricular Cartilage Repair Using Cryogel Scaffolds Loaded
Developing 3D Quantitative Morphometric Characterization of Tissue-Engineering

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Controlling scaffold porosity, pore size and interconnectivity is necessary to facilitate cell migration in tissue-engineering (TE). However, quantification of 3D morphometry is not possible with routine 2D histology, and therefore, advanced methods are required. The goal of this work is to develop imaging protocols applicable to scaffold materials used in cartilage TE to assess their 3D internal microstructure in a physiological environment; specifically, Bacterial Nano Cellulose (BNC)-calcium alginate and silk fibroin.

An evolving strategy for imaging BNC and silk fibroin was implemented using contrast agents that exploited the anionic properties of the alginate in BNC, and the hydrophobicity of silk fibroin. Each scaffold was scanned with micro-computed tomography, μCT (μCT50, SCANCO Medical AG, Brüttisellen, Switzerland) at 45 kVp and 1 μm resolution. The results show that the internal scaffold microstructure of a BNC-calcium alginate scaffold in 0.1 M calcium chloride, quenched in liquid nitrogen and freeze-dried could be adequately visualized and segmented. Comparison with scanning electron microscopy confirmed these structures, and porosity, mean pore size and pore interconnectivity were 75.4%, 48.9 μm and 15 088 mm⁻³, respectively. Similarly, the silk scaffold that was incubated in a solution of Lipiodol® saponified with potassium hydroxide and then transferred to 0.1 M hydrochloric acid could be visualized. Adequate contrast was only achieved in an outer ring of the scaffold due to poor penetration, and methods for improving this are now underway.

This work demonstrates new imaging protocols for quantitative assessment of cartilage TE scaffold materials, outlining future strategies for improvement.
Magnetic Nanoparticle Clusters as a Nanomedicine for Site-Specific Delivery/Imaging

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In this study, we developed novel multifunctional magnetic nanoparticles (MNP) with a folic acid moiety, which can be recognized by many tumors overexpressing folate receptors (FR) in their surfaces. Instead of dispersing iron oxide particles (Fe$_3$O$_4$) in micelles commonly reported, the chemical reaction between the iron oxide particles and PF127 was prepared. These MNP perform multifunctional characteristics, which can be used as magnetic resonance imaging (MRI), optical imaging, as well as chemotherapy agents for targeting drug delivery systems. One-pot synthesized polyacrylic acid-coated iron oxide (PAAIO) was decorated with polymers, PF127 (PF127-PAAIO) or folic acid-linked PF127 (FA-PF127-PAAIO). The hydrophobic PPO segments of PF polymers can be used to encapsulate a therapeutic agent for cancer disease treatments and/or a fluorescent dye for tumor diagnosis.$^1$ PAAIO possess specific magnetic properties upon an exposed magnetic field, which endow them an attractive platform as a contrast agent for MRI. The multifunctional FA-PF127-PAAIO can be used as combined diagnosis and therapy purposes specifically targeting to FR-overexpressed cancer cells. The PF127-PAAIO was served as a control group. According to FTIR and UV absorption spectra, we ensured the successful preparation of the MNPs. The hysteresis curves of SQUID magnetometer analysis also showed that these MNPs were superparamagnetic. The cellular uptake between FA-PF127-PAAIO and PF127-PAAIO in FR-overexpressed KB cells and FR-deficient A549 cells was studied and compared with a flow cytometer and a confocal laser scanning microscope. The in vivo tumor targeting efficacy of the FA-PF127-PAAIO was realized by MRI and an optical imaging technique using KB cells xenograft nude mouse model. The further enhancement of localization of the MNPs at the tumor sites can be manipulated with an assistance of a magnetic field near the tumor sites. Based on all obtained results, we conclude that the surface-modified PAAIO was potentially applied in translational medicine such as magnetofection,$^2$ dual-modality nanoprobe, and theragnostics.
Microfluidics-based gradient makers have the ability to generate large soluble species gradients in just few millimeters. This can lead to interesting applications in drug testing/toxicity assessment due to their ability to construct dose–response curves, in a single experiment, and using a single cell culture. However, it could be interesting to reveal the real-time behavior of cell culture, for which the pH could be selected as target marker. For this reason extended dynamic range pH-sensitive ratiometric nanosensors capable of accurately mapping the full physiological pH range (pH 3.5 to 7.5), have been developed. Nanosensors, with a diameter of 40 nm, were prepared by immobilising pH-sensitive fluorophores and a reference fluorophore in an inert matrix. Accurate ratiometric pH measurements were calculated through determining the fluorescence ratio between the pH-sensitive and reference fluorophores. These nanosensors were successfully embedded in a thin hydrogel layer placed in the main chamber of the sensitive gradient maker device. The sensitive microdevice could have application on revealing the real-time pH variations of cell cultures in response of a chemical gradient of a drug/toxic substance.
Matrix metalloproteinase (MMP) have been known to be essential for successful angiogenesis, specifically to play pivotal role in the onset of angiogenesis primarily by degrading extracellular matrix components. Consequently, direct in vivo imaging of MMP activation may be beneficial for diagnosing angiogenesis-related disease. For effective in vivo imaging, imaging probe should circulate in blood for adequate time. Therefore, we introduced human serum albumin (HSA) having native property of long half-life to overcome the limited circulation time of MMP peptide probe. Three to five MMP peptide probes were covalently conjugated to the HSA (MMP-HSA probes), which showed no cytotoxicity to human dermal fibroblasts and extended circulation time in vitro. MMP-HSA probes provided visualization of MMP activation during angiogenesis process in the murine hindlimb ischemia model using an NIR fluorescence imaging system. MMP-HSA probe showed a marked increase in the NIRF signal from the ischemic tissue within 5 days and showed a maximum at 5 days after ischemia induction. Furthermore, real-time intravascular tracking of MMP activation during angiogenesis in the hindlimb ischemia mouse model is observed. Therefore, this proposed noninvasive approach has significant potential as regards early and accurate diagnosis for angiogenesis-related disease and tracking therapeutic interventions directed at MMP inhibition. Through this, MMP imaging in vivo offers the opportunity that can affect clinician’s decision making and set up a more personalized medicine approach.
Liver fibrosis is tissue's failure to regenerate due to repeated or massive injuries. The role of liver biopsy as the gold standard for fibrosis assessment has recently been challenged due to sampling error and inter- and intra-observer variations. To tackle these issues, we present qFibrosis, an automated quantification system with nonlinear optics microscopy for liver fibrosis assessment that quantitatively characterizes changes in collagen patterns in the course of fibrosis progression. qFibrosis provides a combined score generated from quantification of 87 collagen architectural features. 75 Thioacetamide-induced fibrotic rat samples and 107 biopsy samples from chronic hepatitis B patients were imaged for quantification. qFibrosis can recapitulate the liver fibrosis scoring by pathologists, with area under receiver operating characteristics curve up to 0.99. It is less sensitive to sampling variations, with coefficient of variation smaller than 27%. qFibrosis can significantly predict underestimation of fibrosis scores in suboptimal biopsies as well as from the non-expert pathologist, thus aiding in the correction of intra/inter-observer variation. qFibrosis can also significantly differentiate intra-stage cirrhosis changes that can be monitored for treatment planning and evaluation. Our results indicate that qFibrosis may become a powerful novel tool for precise analysis of fibrosis in basic research of tissue engineering; and a surrogate histological marker for evaluating tissue regeneration and for monitoring anti-fibrotic drug efficacies in clinical practice; or in assessing the tissue remodelling in implants of tissue engineered constructs.
The main goal of this work is to increase objectivity in quantitative analysis of cell outgrowth from neurospheres by proposing an automatic image analysis tool. Neuropheres were obtained from neural progenitors derived from a mouse ES cell line under adherent monoculture and in serum-free RHB-A medium. Neuropheres (150-200 µm in diameter) were seeded on [(Poly-D-lysine)-(Laminin 111)]-coated coverslips (PDL-LN 111) or on TCPS wells coated with Laminin 511 (LN 511). After 24 h of cell culture, samples were fixed, permeabilized, and incubated with DAPI for DNA labeling. Samples were mounted with Fluoromount™ and observed under phase and fluorescent microscopy.

Neurosphere and outgrowth boundaries were estimated using fluorescence and phase contrast microscopy images, respectively. For neurosphere boundary estimation, the fluorescence image was filtered with a Gaussian filter and then segmented using Otsu thresholding. The cell outgrowth boundary was obtained using phase symmetry measure, which was binarized using non-maximum suppression and dilation (Fig.1). The ratio between the outgrowth and neurosphere areas was computed, and the distance between each outgrowth boundary point and the neurosphere boundary was assessed. Results showed for neurospheres seeded on LN 511-coated surfaces significantly higher maximal and average outgrowth distance than those seeded on PDL-LN 111-coated coverslips. By enabling the detection of low-contrasted morphological features, the developed approach enabled the quantitative assessment of cell outgrowth from neurospheres in samples merely processed for DNA fluorescent staining. Measures are in high agreement with manual analysis and the analysis time was reduced in more than 95%.

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The aim of this study was to broaden the relatively small number of qualitative and quantitative data available on the endothelium of human blood vessels by scanning electron microscopy (SEM) imaging and computer based image analysis of the human endothelial microstructure and dimensions. Endothelium of the arterial blood vessels (common iliac artery and hepatic artery) was imaged directly by using SEM. From high quality images, precise information on the microstructure and dimensions of endothelial cells was obtained by using GIMP and Leica QWin image analysis software. The mean endothelial cell width, length, and area of common iliac artery endothelial cells were found to be 13.2±4.1mm, 25.8±8.5mm, and 245.0±115.1mm², respectively. For hepatic artery endothelial cells, mean values of cell width, length, and area were found to be 4.9±1.5mm, 21.9±6.6mm, and 70.7±34.8mm², respectively. Morphology and dimension of the endothelial cells were different depending on the donor, type, and diameter of the arteries due to the effect of blood flow direction and volume rate. The presented method is useful for obtaining quantitative data on human endothelial cells. This study provides a first basis for future studies with larger numbers of samples on morphological changes secondary to pathological conditions, such as hypertension and atherosclerosis. Furthermore, the data may support the development of a template for a novel artificial vascular graft with superior performance.
Establishing an in vivo Analysis Method for High Field MRI to Quantify Perfusion Capacity in DegraPol® foam as Scaffold Material

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Tissue engineered constructs for regenerative medicine are based on scaffolds that are used as a matrix for cells to attach and proliferate, so that these artificial grafts can be implanted at defect sites of tissue. For this purpose they should provide a very good biocompatibility, i.e. they should stimulate the angiogenesis after implantation and guarantee the long-term function and survival of the implanted cells. The transport of oxygen and nutrients is dependent on a homogenously distributed vascular network, especially also in the middle of the implanted scaffold. Current models do not allow easy analysis of angiogenesis of implants in vivo. Therefore, a tool enabling to monitor non-invasively the perfusion capability of these artificial organoids in a cheap, established and reliable in vivo model would add great benefit to the research in this field. In the present study, using the MRI technique we repetitively analyzed perfusion capability of the implants in vivo. Subsequently, capillary networks in scaffolds have been confirmed by histological analysis.

On incubation day (ID) 3.5, egg shells of fertilized eggs were opened. On ID 7, polyurethane scaffolds were carefully placed on top of the chorioallantoic membrane (CAM assay). After one week, on incubation day 14, in vivo high field MRI scans were made focusing the scaffold being meanwhile vascularized by the capillaries of the chick embryo’s chorioallantoic membrane; once not injected (delivering $R_{10}$ values) and then after i.v. injection delivering $R_1$ values. Relaxation rates were correlated to the capillary density scored semi-quantitatively in four succeeding histological sections separated by 1 mm. $R_1$ values were significantly higher than $R_{10}$ values. Capillary density determined by histological analysis correlated well with the relaxation rates measured by high field MRI. High field MRI offers a reliable method to quantify the perfusion capacity of artificial organoids in vivo. This method allows the monitoring of vessel ingrowth into artificial organoids placed on top of a cheap, established and reliable in vivo model, the chorioallantoic membrane of the chick embryo.
A Novel Strategy to Design Scaffolds Mimicking the Polysaccharide and Protein Component Interactions of the Extracellular Matrix

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The extracellular matrix (ECM) has been largely investigated to obtain useful information for the design of tissue engineered scaffolds. However, only a few studies have been carried out for the comprehension of the molecular interactions between the protein and polysaccharide components in different body tissues. In fact, the degree and the type of interactions between these components can influence the secondary structure of ECM proteins, the chemical homogeneity and important properties such as hydrophilicity, mechanical behaviour and microporosity, representing biologically relevant signals for ECM-cells interactions. The knowledge of the interactions between ECM natural polymers and the transfer of it in scaffold development is of fundamental importance for the selection of scaffolds with improved performances. In this work infrared Chemical Imaging analysis was used to evaluate the chemical composition, homogeneity, molecular interactions and protein structural conformation of natural bone and myocardium. The same characterization was performed on gellan gum (GE), gelatin (Gel) and hydroxyapatite (HA) scaffold and GE, Gel and elastin (Ela) scaffold, mimicking the composition of native bone and myocardium, respectively.

Figure 1: Chemical map (a, d), medium spectrum (b, e) and correlation map (c, f) of myocardial tissue (a-c) and GE/Gel/Ela scaffold (d-f).

Chemical maps of both scaffolds showed chemical homogeneity and protein secondary structure similar scaffold to that of the tissue that they will have to replace. Preliminary biological characterization showed a good cell adhesion and proliferation on both materials.

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Auricular Cartilage Repair Using Cryogel Scaffolds Loaded with BMP-7-Expressing Primary Chondrocytes

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Toll-like receptors (TLRs) are a class of pattern-recognition receptors that recognize microbial pathogens and initiate the innate immune defense. Recent studies have shown that the TLR system is not only limited to the innate immune defense against exogenous pathogens. Besides the expression of TLRs on cells of the immune system, it has been shown that TLRs are also expressed on multiple other cell types like endothelial cells where they appear to be involved in inflammation-induced angiogenesis, this being highly important for tissue regeneration and wound repair. The aim of this study was to investigate the role of TLR signaling during angiogenesis and bone tissue regeneration using an established in vitro co-culture model, consisting of outgrowth endothelial cell (OEC) and primary osteoblasts (pOB), and to develop potential approaches to enhance the vascularization process of engineered tissue. In fact, OEC mono-cultures express different TLR receptors like TLR2, TLR3 and TLR4, which could be evaluated using immunofluorescent staining as well as RT-PCR. It could be shown that exogenous ligand-stimulated TLR signaling had a distinct effect on the formation of angiogenic structures in the co-culture of pOB and OEC. Stimulation of co-cultures for 1 week with POLY(I:C), a synthetic ligand for TLR3, resulted in an increase in the formation of microvessel-like structures compared to the control cultures. These results suggest a pivotal role of the TLR signaling pathway during bone tissue repair. Based on the beneficial influence in terms of angiogenic activity, ligand-stimulated TLR signaling might be of therapeutic interest to enhance the bone regeneration process.
Stiffness of Highly Porous Collagen-Based Scaffolds Can Direct the Lineage Specification of Mesenchymal Stem Cells

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Articular cartilage defects within joints eventually result in osteoarthritis, requiring arthroplasty. However, tissue engineering approaches using biomaterials have shown significant promise in healing defects. It is known that the mechanical properties of the extracellular matrix can significantly affect mesenchymal stem cell (MSC) lineage specification. In this context, the aim of this study was to develop collagen-hyaluronic acid scaffolds with properties optimised for chondrogenesis in vitro. The specific objectives were to investigate the effect of scaffold stiffness (0.5, 1.0 and 1.5 kPa) on MSC differentiation and cartilage matrix production in vitro. It was evident that low stiffness (0.5 kPa) stimulated significantly higher MSC early stage gene expression of transcriptional factor, SOX9, in the absence of differentiation factors compared to stiffer scaffolds (1.5 kPa). The stiffest scaffolds (1.5 kPa) stimulated significantly higher RUNX2 gene expression, characteristic of an osteogenic lineage. Stiffness-mediated lineage specification was found to be partly dependent on the contractility of MSCs modulated by an actin-binding motor protein, non-muscle myosin II. Inhibition of non-muscle myosin II led to inhibition of SOX9 and RUNX2 gene expression regardless of scaffold stiffness. In the presence of chondrogenic differentiation factors, it was evident that the most compliant scaffolds supported significantly higher COL2 gene expression by day 14 and cartilage matrix deposition by day 21. This demonstrates the importance of taking into consideration biomaterial mechanical properties in the development of advanced tissue engineering strategies for cartilage repair.
Bone tissue engineering (TE) has developed due to a severe lack of adequate alternatives capable of enhancing healing in critical-sized defects. A major obstacle to be overcome is insufficient blood supply in the implanted tissue. Employing an endochondral ossification (ECO) approach may offer possible solutions to this dilemma as hypertrophic chondrocytes release angiogenic factors such as vascular endothelial growth factor (VEGF) as well as an early marker of osteogenesis, alkaline phosphatase. Blood vessel invasion and subsequent mineralisation of a cartilage precursor are the result of this process. In this context, the aim of this project was to develop an in vitro model of ECO using collagen-based scaffolds that can support tissue-engineered cartilage formation. Collagen-glycosaminoglycan (CG) and collagen-hydroxyapatite (CHA) scaffolds were seeded with rat mesenchymal stem cells (rMSCs) in the presence of chondrogenic factors. Sulphated GAG quantification and histology demonstrate that both scaffolds support cartilage matrix deposition, with CG scaffolds promoting significantly greater levels of cartilage-like matrix deposition (p<0.01) by day 42. COLX and VEGF gene expression were evident in both scaffold groups, with CG supporting significantly higher COLX expression. There was increased mineralisation with time up to 42 days in both scaffolds, however, the CHA scaffold contained significantly higher calcium (p<0.01). This study demonstrates the potential of these biomimetic scaffolds as cartilage precursors to mimic the process of ECO for bone repair applications. In vivo assessment of this model is currently ongoing to assess the healing of a mid-diaphyseal femoral defect.

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Biomimetic ECM Constructs Based on Collagen-heparin Glycosaminoglycan for Bone Tissue Engineering

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New strategies in regenerative medicine and tissue engineering include the development of biomaterials and scaffolds able to mimic the natural extracellular matrix (ECM) and modulate regenerative processes. Bone ECM is a complex ordered hierarchical structure, as a result of the assembly of collagen fibrils at several length scales. The fibrous bed of the complex bone ECM, which supports and regulates cell activity, has inspired many researchers to develop biomimetic scaffolds based or functionalised with native collagen and glycosaminoglycans. This work deals with the design and realization of bioactive surfaces based on collagen type I and heparin. Interfaces were prepared through controlled deposition of Collagen-Heparin macromolecules assembled using a layer-by-layer (LbL) technique on poly (l-lactic acid) (PLLA) constructs for tissue regeneration. These constructs may support functional tissue formation by controlled growth factors linked to the GAGs (heparin) delivery by stimulating scaffold vascularization, inducing cell organization, providing space for the perfusion of nutrients throughout the scaffold, influencing the human mesenchymal stem cells (hMSCs) activity and improving the mineralization. Porous scaffolds prepared by the Thermally Induced Phase separation (TIPS) technique and bio-functionalized by the LbL technique with Heparin-Collagen coatings were evaluated in vitro using hMSCs. Results suggest that PLLA constructs multilayered with Heparin-Collagen macromolecules regulate the different stages of hMSCs adhesion, differentiation and interaction with other proteins and biomolecules. Increasing the number of heparin-collagen layers on 2D PLLA constructs slowed down hMSCs growth and differentiation in the short-term while enhanced osteogenic activity after 28 days. However, in 3D scaffolds it was observed that increasing the numbers of collagen-heparin multilayers stimulated cell growth and decreased hMSCs differentiation into bone at long term. These results highlight that ECM based regulation of cell activity is a complex interaction between the type and molecular organisation of biomolecular ligands and their microscale spatial distribution.
Preconditioning Periosteal Cells by Inactivation of the Phd2 Oxygen Sensor Improves Their Bone Forming Capacity in a Large Segmental Bone Defect

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The hypoxic environment that a bone tissue engineered construct encounters upon implantation is deleterious for cell survival and thus impedes bone repair. We hypothesized that activating the hypoxia inducible factor (HIF)-pathway in the implanted cells will not only promote angiogenesis, but also facilitate metabolic reprogramming and hereby reduce cell death.

We genetically inactivated the Phd2 oxygen sensor in murine periosteum derived cells (Phd2<sup>low</sup> mPDC) which resulted in HIF-1α stabilization in normoxic conditions. Activation of the HIF-pathway resulted in increased production of the angiogenic factor VEGF. In addition, mRNA levels of key enzymes in glycolysis and mitochondrial metabolism were altered, indicative of metabolic rewiring. Compared to control mPDC, implantation of Phd2<sup>low</sup> mPDC in a large segmental defect in the mouse tibia resulted in the formation of significantly more bone, surrounding islands of bone marrow. Intriguingly, control mPDCs formed bone only at the periphery of the scaffold, whereas Phd2<sup>low</sup> mPDC formed bone also in the centre, a site considered to be more hypoxic. Additionally, the number and size of the blood vessels was increased and they were closely associated with sites of bone formation.

In conclusion, genetic inactivation of Phd2 activates the hypoxia signaling pathway in periosteal cells, improving their potential to form bone and attract blood vessels in vivo.
The Interplay between Chondrocyte Redifferentiation Pellet Size and Oxygen Concentration

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Chondrocytes dedifferentiate during monolayer expansion. Aggregation of the expanded cells into pellets, in the presence of induction factors, facilitates their redifferentiation. Typically 1x10⁵ - 5x10⁵ chondrocytes are aggregated, resulting in pellets having diameters ranging from 1-2 mm. However, diffusion of metabolites over such length-scales is inefficient, resulting in radial tissue heterogeneity. Herein we demonstrate that the aggregation of 2x10⁵ human chondrocytes into micropellets of 166 cells each, rather than into larger single macropellets, enhances the chondrogenic redifferentiation. We describe the fabrication of a microwell surface for the large-scale production of micropellets. Sulfated glycosaminoglycan (sGAG) production and collagen II gene expression in chondrocyte micropellets increased significantly relative to macropellet controls, and redifferentiation was enhanced in both macro and micropellets with the provision of a hypoxic atmosphere (2% O₂). It was also demonstrated that micropellets could be assembled into larger cartilage tissues. Our results indicate that micropellet amalgamation efficiency is inversely related to the time cultured as discreet microtissues. In summary, we described a micropellet production platform that represents an efficient tool for studying chondrocyte redifferentiation and demonstrated that the micropellets could be assembled into larger tissues potentially useful in cartilage defect repair.
In vivo Ultrasructural and Functional Maturation of Renal Organoids Engineered from Embryonic Kidney Cells

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Chronic kidney disease is a major cause of morbidity and mortality: the shortage of transplantable organs greatly enhances the need for tissue-engineered alternatives. We have shown that organoids constructed in vitro from embryonic kidney cells can integrate into living recipients. Here, we assessed sieving function and physiological response of erythropoietin (EPO)-producing organoids. We dissociated enzymatically E11.5 mouse kidneys, centrifuged single cell suspensions, and cultured the pellets (5d). Then, renal organoids were implanted beneath the renal capsule of a rat host (euthanized at 3w). EM showed glomeruli in various developmental stages, with glomerular capillary walls covered by podocytes. In addition to immature junctions, foot processes had differentiated morphology and filtration slit diaphragms. Normal-appearing slits were rare, but the proportion of slit diaphragms therein was comparable to that found in adult glomerulus (80% vs 91%, respectively). We tested the physiological function of nephrons by injecting FITC-, RITC- or TRITC-conjugated dextrans (10, 77 or 155 kDa, respectively) into host blood system. Proximal tubule cells concentrated 10 and 77 kDa dextran from lumen, as shown by colocalization with megalin receptor for endocytosis. No TRITC-dextran was found in graft tubular lumen, as in the host, implying efficient ultrafiltration. Real Time PCR analysis of grafted tissue showed a dramatic increase (62.5 fold) in expression of mouse EPO mRNA in response to anemia due to blood withdrawal. These results show that a tissue-engineering protocol can be successfully applied to single embryonic kidney cells, to grow graft tissue for use in translational studies.
High Throughput Generated Chondrocyte Micro-aggregates Boost Neo-cartilage Formation In Vitro and In Vivo

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Successful cell-based tissue repair strategies rely heavily on cell performance. Single cells laden hydrogels have shown great promise for cartilage repair. However, cartilage's natural regenerative response is partly mediated by forming chondrocyte clusters. We hypothesized that neocartilage formation could be improved by seeding hydrogels with bio-inspired chondrocyte micro-aggregates instead of single chondrocytes. Chondrocyte micro-aggregates were produced in high throughput using a custom build micro-mold. Chondrocyte micro-aggregates' morphology, stability, gene expression and chondrogenic potential were investigated and compared to single cells. Additionally, the cartilage formation of both single cells and micro-aggregate laden Dextran based hydrogel was compared in vitro and after subcutaneous implantation in nude mice. Micro-aggregates were produced with highly controlled size, stability and viability. Microarray analysis revealed an overall increase in expression of cartilaginous genes in micro-aggregates. Indeed, qPCR validated that COL2A1 and ACAN mRNA levels were significantly higher and MMP 1, 9 and 13 mRNA levels were significantly lower in micro-aggregates of 50 or 100 cells compared to single chondrocytes. Histological analysis of Dex-TA hydrogels laden with 100 cell micro-aggregates demonstrated enhanced cartilage formation compared to single cell laden hydrogels, both \textit{in vitro} and \textit{in vivo}. High throughput generation of chondrocyte micro-aggregates represents an effective technique to accelerate neocartilage formation in biomaterials and may boost neocartilage regeneration in cell-based tissue repair strategies.
Intra-Articular Adipose Derived Stromal Cell Delivery Contrasts Osteoarthritis Progression in an Experimental Rabbit Model

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Increasing incidence of Osteoarthritis (OA) and the aging population coupled with insufficient therapies has led to focus on the potential of stem cells as novel strategy for cartilage repair. This work is aimed to investigate the efficacy of an intra-articular Adipose derived Stromal Cell (ASC) injection in a rabbit experimental model. The induction of OA was performed surgically through Anterior Cruciate Ligament Transection to achieve at 8 weeks a mild grade of OA. 2x10⁶ and 6x10⁶ autologous rabbit ASC were administered into the affected knee joints. Fluorescent labeled ASC were used to determine the bio-distribution of the cells into the joint early after implantation. Cartilaginous and synovial tissues were assessed using histological evaluations by Laverty’s scoring systems. Cartilage matrix molecules, catabolic and inflammatory markers were determined by immunohistochemistry. Assessment repair was carried out at 16 and 24 weeks. Labeled-ASCs were detected in the synovial tissue and meniscus. Histological assessments showed a positive contribution of ASC in the healing of cartilage with significant statistical evidences particularly with the lowest cell dose. Immunohistochemical evaluations confirmed the repair processes exerted by ASC on synovial and cartilaginous compartments. Our data reveal a healing capacity of ASC in promoting cartilage and menisci repair and attenuating inflammatory events in synovial membrane inhibiting OA progression. On the basis of the local biodistribution findings, the benefits obtained by ASC treatment could be due to a trophic mechanism of action by the release of growth factors and cytokines.
Pre-clinical Safety Study to Evaluate Potential Biodistribution and Tumorigenicity of Human Chondrocyte Spheroids (ATMP) in the NSG Mouse Model

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Articular cartilage lesions can be treated with an Advanced Therapy Medicinal Product (ATMP) consisting of human chondrocyte spheroids (co.don chondrosphere\textsuperscript{®}). Authorisation of ATMPs by authorities (EMA) requires non-clinical GLP studies. To prove the safety of this ATMP in terms of biodistribution (Bd) and tumorigenicity (Tg) a GLP study was performed. Chondrocyte spheroids derived from female human patients were subcutaneously implanted into the backs of immunodeficient NOD.Cg-Prkdc\textsuperscript{scid}Il2rg\textsuperscript{-1Wj}/SzJ (NSG) mice (Bd and Tg: for each study 5 patients á 8 mice). In addition, for Tg study, two reference groups (NIH-3T3 and human Caco-2 carcinoma cells) were analyzed. The Bd group was sacrificed one month, the Tg group six months post implantation or earlier, in case of apparent tumor formation. Bd was analyzed by Multiplex PCR and immunohistochemistry (IHC) using an HLA-ABC antibody. Tg was studied by palpation, macroscopic evaluation and histology (HE). In case of a tumor, a detection of HLA-ABC followed to analyze a possible origin from the implant. After screening of 2556 cryosections by IHC, no signs of migration were observed. Only 5 of 104 samples very close the human implant were positive in the RT-PCR. In none of the mice organs human female DNA could be found. No human tumor formation was found in mice. Two samples verified as tumors were shown to be of murine origin due to the lack of HLA-ABC expression. All mice of the reference groups developed tumors. This study supports the safety of huChon in terms of biodistribution and tumorigenicity since there was no migration of human chondrocytes or evidence for tumor formation caused by human chondrocytes in the NSG mouse model.
Obtaining a sufficient number of cells \textit{ex vivo} for tissue regeneration, which are appropriate for cartilage repair, requires improved techniques for the continuous expansion of chondrocytes in a manner that does not change their innate characteristics. Rapid senescence or dedifferentiation during \textit{in vitro} expansion results in loss of chondrocyte phenotype and the formation of fibrous cartilage replacement tissue, rather than hyaluronic cartilage, after transplantation. As demonstrated in the current study, wild-type p53-inducible phosphatase (Wip1), a well-established stress modulator, was highly expressed in early-passage chondrocytes, but declined rapidly during \textit{in vitro} expansion. Stable Wip1-expressing chondrocytes generated by microporation were less susceptible to the onset of senescence and dedifferentiation, and were more resistant to oxidative stress. The increased resistance of Wip1 chondrocytes to oxidative stress was due to modulation of p38 mitogen-activated protein kinase (MAPK) activity. Importantly, chondrocytes expressing Wip1 maintained their innate chondrogenic properties for a longer period of time, resulting in improvements in cartilage regeneration after transplantation. Chondrocytes from Wip1 knockout (Wip1	extsuperscript{-/-}) mice were defective in cartilage regeneration compared with those from wild-type mice. Thus, Wip1 expression represents a potentially useful mechanism by which a chondrocyte phenotype can be retained during \textit{in vitro} expansion through modulation of cellular stress responses.
Mesenchymal Stem Cells (MSCs) have the capacity to form cartilage. Cartilage repair \textit{in vivo} using MSCs might be influenced by other cells in the joint such as macrophages residing in the synovium. Macrophages can be divided into pro-inflammatory macrophages (M1) or anti-inflammatory/repair macrophages (M2). Our goal was to investigate the influence of M1 and M2 macrophages on MSC chondrogenesis to know how diseased conditions influence MSC differentiation. Monocytes were treated with IFNγ and LPS (M1 differentiation) or IL4 (M2 differentiation) for 72 hours followed by removal of the stimuli and culture in chondrogenic medium. Conditioned chondrogenic medium was harvested and used for MSC chondrogenesis experiments: MSCs were chondrogenically primed for 14 days followed by 48 hours of incubation in 20% conditioned medium. Conditioned medium was analysed for IL6 and CCL18. Conditioned medium contained IL6 after monocyte treatment with IFNγ and LPS, or CCL18 after monocyte treatment with IL4. M1 conditioned medium decreased \textit{Col2} 4.5 times and \textit{Agcn} 6 times compared to MSCs cultured in normal chondrogenic medium. M2 conditioned medium had no effect on \textit{Col2} and \textit{Agcn}. Macrophages, and more specifically pro-inflammatory macrophages, can influence MSC chondrogenesis negatively. This might have implications when stimulating cartilage repair using MSCs \textit{in vivo}. 

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A major challenge in cartilage tissue engineering is to define an appropriate cell source to obtain cells for the reconstruction of cartilage defects. We aimed to compare the functional properties of constructs produced by several cell sources in vitro and in vivo.

Human chondrocytes from ear (EC), nose (NC), and articular joint (AC), and MSCs from bone marrow (bMSC) and adipose tissue (aMSC) were expanded and cultured in alginate to assess chondrogenesis. After 5 weeks, constructs were implanted subcutaneously in athymic mice (8 weeks). Before and after implantation, matrix components were measured using biochemical assays. Signs of hypertrophy and subsequent bone formation were evaluated with RT-PCR and histology, and mechanical properties were measured in simple compression.

ACs have a superior chondrogenic capacity in vitro, as assessed by gene expression and GAG production (p<0.001), and compared to residual cell sources. However, after implantation, ACs retained their matrix but did not further increase it. On the contrary, ECs and NCs continued producing matrix in vivo leading to more GAG (p<0.001). The increased GAG content in EC and NC containing constructs did not reveal a higher elastic modulus. Although the matrix formed by bMSCs encapsulated in alginate highly expressed collagen type X and alkaline phosphatase in vitro, they did not mineralize in vivo.

This work indicates that ECs and NCs are most suitable for cartilage tissue engineering in vivo. The inability of ACs to increase cartilage matrix in vivo may be due to a loss of chondrogenic capacity in the absence of mechanical loading or growth factor stimulation.
Osteogenic Potential of Mesenchymal Stromal Cells-adipose Derived: a Preliminary Study

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The novelty in mesenchymal stem cell research has been represented by the adipose tissue as promising source of mesodermal derived-multipotent cells, called adipose stromal cells (ASCs) [Zuk \textit{et al.} 2001]. Adipose tissue -normally discharged as medical waste- can be harvested with a minimally invasive liposuction, and ASCs could overcome important constraints such as harvest site pain, morbidity and risks of infection.

The aim of this study was to analyze the influence of different human adipose harvesting sites on ASCs yield, proliferation, stemness, characterization and osteogenic potential when cultured in differentiating condition. 18 specimens were collected by liposuction from 14 subjects (12 females and 2 males) with different age, body weight, height and body mass index: 6 samples were obtained from abdominal area, 5 from trochanteric area and 7 from breast.

Surface characterization showed the typical mesenchymal CD pattern (CD44, CD73, CD90, CD105) and negative expression of endothelial and hematopoietic markers (CD31 and CD45). No significant differences among the harvesting sites were found for ASCs characterization, yield and stemness, collagen type I gene activation, alkaline phosphatase synthesis and mineralized nodules formation. Abdomen derived ASCs showed lower proliferation values than breast and trochanteric ACSs (F=3.56, \textit{p}<0.05) and higher values of RUNX2 (F=4.22, \textit{p}<0.05) and TGF\textsubscript{\beta}1 (F=3.86, \textit{p}<0.05).

These preliminary results demonstrate that ASCs, regardless to harvesting sites and patient characteristics, could be a viable and abundant cell source, able to differentiate into osteoblasts and to be useful in orthopedic regenerative medicine.
Bioengineered Tumoral Microtissue: An *In Vitro* 3D Platform to Investigate Cell-ECM Interaction and Cancer Development

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Cancer is a complex pathology and 2D cell model are not able to reproduce tumoral morphology, cell-cell and cell matrix interactions. Moreover animal models do not adequately reproduce features of human tumors, drug therapeutic responses and stem cell differentiation. *In vitro* 3D tissue models bridges the gap between traditional cell culture and animal models. The aim of this project is the realization of tumoral heterotypic microtissue, by seeding both stromal and tumoral cells on biodegradable microscaffold. In particular dynamic co-cultures of cancer associated fibroblasts (CAF) or their control (CAF522) with pancreatic cancer cell (PT45) on gelatin microcarriers are performed in spinner flasks for 12 days during which the cells develop a tissue structure. At the same time also CAF, CAF522 and PT45 single culture are grown to investigate the differences. Cells were counted and stained according to hematoxylin/eosin and trichromic Masson procedure. Microtissues are characterized by IF and confocal microscopy. After 12 days exogenous scaffold disappeared and new collagen has synthesized. Histological analysis showed similar features between 3D tissue and human cancer tissue. Stroma can play important roles in supporting or even inducing tumorigenesis, and 3D heterotypic tumor model realized is a good candidate to study the specific roles of stroma in tumor development, progression, local invasion. Moreover our model may help to make drug development safer and more efficient and reduce research and development expenses. Additionally, they represent promising models for individualized oncologic therapy by revealing new insights into mechanisms of organogenesis and expression of malignancy.
Investigations in regenerative medicine and biomaterials sciences have the aim to restore structure and function of damaged tissue/organs. The goal of this medical approach is the creation of appropriate cell/biomaterials construct, finding the way to cure previously untreatable injuries and disease. In this context, the current work describes the production of composite biopolymer scaffolds for bone tissue engineering applications. In particular, we focused on the development of microfibrous systems mimicking the in vivo microenvironment through the enhancement of cell–cell and cell–matrix interaction/signaling. Microfibers were constituted of blends of alginate (Alg), gelatin (Gt) and an extracellular matrix obtained from porcine urinary bladder matrix (UBM). The use of composite materials had the aim to overcome the limitations of the single biopolymer and optimize, at the same time, viability and differentiation of various embedded cells, including: human mesenchymal stem cells, osteoblasts and chondrocytes. Microfibers were produced by an extrusion method developed in our lab based on a multinlet microfluidic chip, allowing the simultaneous control of different biomaterial solutions. The cells embedded in typical microfibers, constituted of 2.25% Alg, 2.25% Gt and UBM 0.5% were cultured in control and differentiating media for different length of time, showing high viability and specific alignments. Moreover, the analysis of gene expression, extracellular matrix deposition, histological and morphological observations revealed that composite microfibers supported the osteogenic differentiation without the presence of the typical osteogenic inducers currently used for conventional 2D culture.
In-vitro and in vivo Biocompatibility of Covalently Cross-linked Chitosan Fibers for Bone Tissue Engineering Applications

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Introduction: Chitosan, an amino polysaccharide is biocompatible, biodegradable but suffers from mechanical instability. To overcome this instability, it is cross-linked by various agents. However, most of the covalent cross linking agents are toxic in nature and are not preferable for biomedical applications. The aim of this study is to crosslink chitosan fibers with non-toxic biocompatible agent.

Experimental: Chitosan fibers were prepared in sodium hydroxide (NaOH) by wet spinning. They were then cross-linked using 2,5-dimethoxy-2,5-dihydrofuran (DHF). Physicochemical characteristics (FTIR, biodegradation and mechanical) were carried out to evaluate the effect of crosslinking. In vitro compatibility was investigated using cell morphology and MTT analysis. In vivo experiments were carried out.

Results: The FTIR analysis revealed presence covalent bonding between chitosan molecules and DHF in chitosan-DHF fiber. Mechanical strength and biodegradability improved upon crosslinking. Finally, in vitro and in vivo results showed that the fibers were biocompatible.

Conclusion: The chitosan–DHF-iodine cross linked fibers were biocompatible and is suitable for various tissue engineering applications.
Structure and Function of Extracellular Fat Matrix Generated by Human Mesenchymal Stem Cells

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Obesity is a global challenge for health care. For its reduction, many studies were performed, but significant results have not yet been achieved. Main components of adipose tissue are fat cells and extracellular matrix. However, so far obesity research was focused on fat cells. Our aim is to better know ECM structure and function. Human mesenchymal stem cells were adipogenic differentiated for 15 days, and histology, antibody staining and genome wide expression profiling was performed to study the structure and underlying genetic machinery of ECM. Adipogenesis was verified by Oil red O staining of lipid droplets and by qPCR of adipogenic markers PPARG and FABP4. Antibody staining demonstrated collagen type I, II, IV filaments inside the ECM. In addition, alkaline phosphatase activity revealed the ossified nature of tubules within a cytoskeleton framework with hexagonal structure and tubules interwoven in a crisscross manner. Regarding the genetic machinery, ADAMTS9, COL4A1, GPC1, -4, ITGA7, ICAM3, SDC2 and TIMP4 were significantly up-, and ADAMTS5, BGN, CLDN11, COL4A1, ITGA2, -4, ITGB1, -8, LAMA3 and TIMP2, -3 downregulated. Further genes associated with ECM formation, structure, function and linked signaling pathways were regulated. Adipogenic cultures were cultivated in adipogenic propagation medium, resulting in a loss of collagen antigens and lipid droplets after 12 days. Lipid droplet free ECM was adipogenic stimulated again. Strikingly, lipid vacuoles within the ECM were filled with droplets after 5 instead of 15 days. This indicates a main role of ECM in adipogenesis and highlights the importance of our approach to further analyze structural and functional interactions of fat cells and ECM not only for obesity research.

Figure Extracellular matrix of mesenchymal stem cells differentiated adipocytes

The collagen types I (A) and II (B) staining confirmed the presence of interwoven collagen filaments within ECM, as more prominently shown after Oil Red O counter staining (E). Adipogenic cultures were cultivated in adipogenic propagation medium, where they loss lipid droplets after 12 days, leaving bare ECM without lipid droplets and negative for collagen type I and II filament-antigens (C and D). Lipid droplet free ECM (F) was verified by alkaline phosphatase staining for ossified character (G), and then stimulated again adipogenically. Strikingly, lipid vacuoles within the ECM were filled with droplets after 5 (H) instead of 15 days (A and B). Bar: A, B: 20 μm, C-H: 10 μm.

Key words: Obesity, adipogenic differentiation, extracellular matrix, dedifferentiation, re-differentiation, mesenchymal stem cells
Label Retaining Cells in Bladder for Urogenital Tissue Engineering

Stem cells (SC) are able to self-renew and to differentiate into various cell types. During life, adult SC (ASC) are involved in the maintenance and repair of adult tissue. In regenerative medicine, the availability of donor tissue is often limited. The combination of (A)SC with biomatrices may provide alternative approaches. Urothelium is composed of basal, intermediate, and superficial cells. It has been hypothesized that the SC are located in the basal cell layer. The identification and selection of urothelial SC may be crucial to tissue engineer a bladder wall. The aim of this study was to identify urothelial SC by identifying slow-cycling BrdU/EdU retaining cells (RC) in parallel with SC markers.

Pregnant mice received BrdU/EdU (50 µg/g) from embryonic day 8 to 13 for 2 consecutive days. Tissue from embryonic 17 days, 5 days postnatal (p5), 1, 3, and 6 months old mice were harvested and examined by immunohistochemistry (IHC). SC markers used to phenotype RC were CK7, CK14, CK20, p63, ki67, CD44, Trop2, CD117, and CD133.

RC decreased with ageing (p5 13% to 6 months old 1%). CK14 and CD117 expression decreased with ageing too (p5 2.68% to 1 month old 0.5%). RC of pups were distributed in all cell layers (CK7⁺, CK14⁺, CK20⁺, and p63⁺) whereas RC in mother mice were only present in the basal cell layer. RC were Ki67⁻ and CD133⁻. RC were Trop2⁺ and CD44⁺ but they were not SC markers in bladder. RC were concentrated in the trigone and colony forming assays confirmed that isolated urothelium from the trigone form more colonies than cells from other areas.

RC were reduced over time and were specifically located in the trigone suggesting they may represent a SC pool in the adult bladder. These cells may be valuable for tissue engineering purposes of bladder tissue.
Bone-Marrow-Derived Mesenchymal Stem Cells Have a Superior Capacity to Enhance Chondrogenesis in Co-Culture with Chondrocytes Compared to Adipose-Tissue-Derived Mesenchymal Stem Cells

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Co-culture of chondrocytes and mesenchymal stem cells (MSCs) is considered a promising strategy for the repair of cartilage defects. However, whether the role of MSCs is dependent on their origin or is a more general MSC-characteristic is not yet understood. The goal of this study was to test whether the addition of MSCs from either bone marrow (bMSCs) or adipose tissue (aMSCs) would enhance stable cartilage formation in vitro and in vivo.

Bovine articular chondrocytes (AC; N=3 pools), human bMSCs (N=2) and aMSCs (N=2) were encapsulated in alginate discs as single-cell-type populations or as co-cultures of both ACs and bMSCs or aMSCs. Constructs were either cultured in vitro for 3 weeks or directly implanted subcutaneously in nude mice for 8 weeks. Glycosaminoglycan (GAG) content was analyzed biochemically and mechanical properties were measured in simple compression.

The production of GAG was enhanced during in vitro co-cultures of bMSCs and ACs compared to single-cell-type populations (p<0.05). This beneficial effect was not observed in aMSC/AC-cultures. In vivo, GAG content in co-culture conditions was similar to the 100% AC-conditions, irrespective of the MSC source. Mechanical properties did not significantly differ between the groups.

BMSCs showed an increased chondrogenic or chondro-inductive capacity compared to aMSCs through in vitro co-culture, indicating that the role of the MSCs is dependent on their origin. Future studies need to clarify how the interaction between MSCs and ACs may lead to improved cartilage matrix formation.
Towards Vascularization of Thick Tissues – Novel Co-culture System of Primary Human Mesenchymal and Endothelial Cells


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Simulating angiogenesis *in vitro* is of particular interest both for developmental biology and tissue engineering as adequate blood vessel formation is paramount for normal development of tissues *in vivo* as well as survival of engineered scaffolds. Here we present a novel co-culture system, based on two methodologies, plastic compression (PC) of collagen and co-culture of mesenchymal and endothelial cells. PC is a technique that allows cell-independent but cell-friendly fabrication of dense, tissue-like collagen constructs. Recently a standard method of PC has been modified to allow simultaneous fabrication of multiple constructs. This technique has already been employed for ocular TE purposes. It is known that co-culture of human mesenchymal cells and human primary endothelial cells without addition of angiogenic growth factors leads to formation of capillary-like structures. This co-culture system has been applied to several 3D biomaterials and shown to lead to capillary-like structure formation *in vitro* as well as rapid inosculation with the host blood vessels *in vivo*. In this study constructs containing primary human fibroblasts and osteoblasts were prepared in a multi-well format and co-cultured with human microvascular endothelial cells with the acellular PC collagen supportive matrix and 2D controls. Construct morphology was analysed at different time-points and the level of VEGF in the culture medium was monitored. It has been shown that this novel co-culture system leads to a dramatic change in HDMEC morphology within 1 week of co-culture, demonstrating vessel-like patterns. Endothelial cells on acellular PC control retained a cobble-stone morphology. These vessel-like structures were stable over 4 weeks in culture. In addition, EC on acellular PC constructs remained viable and showed no signs of apoptosis during long-term culture compared to 2D monoculture controls. In conclusion, we have developed a novel co-culture system of dense matrix-bound human stromal cells and human microvascular cells with the distinct advantages over 2D co-culture system. Additionally, these constructs can be easily handled and provide an exciting opportunity to assemble thick multilayered tissues for TE applications.
Potential of Resveratrol on Mesenchymal Stem Cell Proliferation and Differentiation

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Resveratrol (RSVL) has a wide range of pharmacological properties including the promotion of bone formation. In the present study, we investigated in vitro effects of RSVL on cell proliferation and osteoblastic differentiation in human mesenchymal stem cell cultures (MSC). For this purpose, MSCs (1x10^5) were inoculated in 6-well plates. RSVL was used at various concentrations (0.1, 10, 20, 40 μM) for 1, 4, 7 and 14 days. We determined the proliferation capacity and viability using WST-1, and apoptosis level using AnnexinV-PI staining with flow cytometer. We observed that RSVL (10 and 20 μM) (2.74±0.03 and 3.07±0.04) (p<0.001) stimulated the cell proliferation while high doses (40 μM) (1.48 ± 0.09) (p<0.001) were stopped compared the control group (2.54 ± 0.04) in WST-1 proliferation assay. The expression of Alkaline phosphatase (ALP) was detected in the early osteogenic induction on 21st day and the calcified nodules were observed by Alizerin Red S staining. We investigated that RSVL dose dependently increased the ALP activity of MSCs to a value 2.5 times the control activity at 40 μM (1.616 ± 0.03) compared to control (0.598 ± 0.01) (p<0.001). We observed a significant increase in expression of the gene BMP2 and BMP4 with Realtime RT-PCR analysis after osteogenic differentiation with RSVL. In conclusion, RSVL directly stimulates both cell proliferation and osteogenic differentiation dose dependently. Increased expression of BMP2 (0.71 ± 0.06) and BMP4 (0.32 ± 0.01) gene shows us that RSVL has a direct stimulatory effect on bone formation in cultured osteoblastic cell in vitro compared to control for BMP2 (0.18 ± 0.01) and BMP4 (0.02 ± 0.001) (p<0.001). Presumably, resveratrol is a useful tool in the prevention of and therapy for osteoporosis.
Tenogenic Differentiation of AD-MSCs via Simulation of Tenocyte with Manipulated Enlongated Cell Morphology

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Based on our previous work, we hypothesize that elongated morphology (EM) serve as a niche factor for adipose derived mesenchymal stem cells (AD-MSCs) into tenocytes. Primary mouse AD-MSCs were isolated from harvested adipose tissue with cell sorting using \textsuperscript{lineage-}/CD34\textsuperscript{+} as a marker Cells were cultured either on smooth (Group A) or microgroove silicone membrane (Group B). Realtime PCR was employed to investigate the effect on EM mediated AD-MSCs tenogenic differentiation and its mechanism. \textsuperscript{lineage-}/CD34\textsuperscript{+} cells exhibited stronger abilities in proliferation, clone formation and multi-differentiation than others subpopulations, indicating the enriched MSCs in this subpopulation. Compared to Group A, Group B cells exhibited an EM with significantly increased expression of tenogenesis markers including tenomodulin, scleraxis, collagen I, III and VI, etc. Importantly, the gene expression of adiopogenic and chondrogenic markers were significantly decreased as opposed to those of Group A (p<0.01). In addition, EM also led to increased RhoA-GTP activity. Furthermore, cytochalasin D could inhibit EM induced tenogenic differentiation, whereas could relatively enhance tenogenic differantiation. Moreover, EM failed to induce tenogenic differentiation of Group B cells derived from scleraxis KO mice. In conclusion, EM can induce tenogenic differentation of purified ASCs via integrin and scleraxis.
Effects on Proliferation and Differentiation of Human Mesenchymal Stem Cells by Lyophilized Extracts from Thrombocyte Concentrates

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Thrombocytes play a fundamental role in hemostasis and are a natural source of growth factors in tissue regeneration. To examine the potential of thrombocytes for therapeutic application in regenerative medicine, we tested growth factor composition of lyophilized extracts of thrombocyte concentrates (TC) and their effects on human mesenchymal stem cell (MSC) proliferation and differentiation in vitro. Therefore, TC were washed, osmotically lysed and subsequently lyophilized. The presence of different growth factors (i.e. EGF, IGF1, PDGF-AB, TGF-β1, FGF2, and VEGFA) was tested by ELISA. Human MSC in vitro were exposed for up to 35 days to TC extracts in unstimulated conditions (US) and after specific osteogenic stimulation (OS, with dexamethasone, β-glycerophosphate, ascorbic acid) and adipogenic stimulation (AS, with dexamethasone, insulin, IBMX, indomethacin). After 14 and 35 days, we analyzed cell number, metabolic activity and differentiation degree of MSC. Most of the growth factors tested were detected in relevant amounts. However, concentrations differed strongly in dependency on the extraction procedure. Proliferation of human MSC was increased in a TC concentration-dependent way. In contrast, the osteogenic and adipogenic differentiation degree was significantly decreased by permanent exposure of MSC to TC extracts in vitro. Thus, processing of TC extracts allows concentration of biologically active growth factors which could be utilized for autologous therapies in regenerative medicine. Long-term exposure (up to 35 d) sustained the “stemness” of MSC in vitro albeit exposure to specific differentiation compounds. (Financially supported by the federal state Mecklenburg-Vorpommern and the EU)
SOX9 Recovery from Microtia Chondrocytes Cultured onto a CTS Based Polymer


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Successful tissue engineering (TE) therapy for elastic cartilage regeneration is based on the combination of three elements: cells, molecular signals and biomaterials. Long-term maintenance of chondral phenotype represents a major challenge for such therapy. Normal chondrocytes undergo dedifferentiation process when cultured in monolayer; however, little is known about phenotype maintenance when we use human microtic tissue as a cell source. Objective To evaluate elastic chondrocytes from microtia patients in order to determine the maintenance of phenotype and suitability for TE applications. Methods Tissue was obtained from ear reconstruction procedures. Chondrocytes were isolated and expanded in vitro. After P1, cells were seeded onto CTS-PVA-ECH polymers and cultured for 20 days. Cell viability was assessed. Specific cartilage molecular markers ELN, COL2, ACAN and SOX9 were detected by PCR. Results Elastic chondrocytes displayed regular growth and proliferation in vitro. Calcein assays revealed 87% of cell viability when seeded onto the CTS-PVA-ECH polymer and after 20 days in culture. ECM proteins were previously determined. PCR analysis confirmed the presence of elastic cartilage markers such COL2 and ACAN through the in vitro expansion phase and after 20 days seeded onto polymer. It is noteworthy that the transcriptional factor SOX9 was barely expressed in PC and recovered its expression when the cells were seeded onto the polymer. Discussion Even though dedifferentiation process was described in culture conditions, microtic auricular chondrocytes maintained phenotype. SOX9 expression along with COL2 and ELN onto CTS-PVE-ECH polymers suggest that microtic tissue represents a suitable source of cells for TE.
Identification of Putative Reference Genes in Mesenchymal Stromal Cells from Osteoarthritic Donors

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Osteoarthritis (OA) is a frequently occurring joint-degenerating disease. Mesenchymal stromal cells (MSCs) are key cellular components for musculoskeletal tissue engineering strategies. Recent data suggest that MSCs are involved in the development of OA. Quantitative reverse transcription polymerase chain reaction (qRT-PCR) is commonly used to detect changes in mRNA expression levels. In this context normalization using stably expressed reference genes is crucial. However, suitable reference genes for the molecular evaluation of MSCs derived from donors exhibiting advanced-stage OA as a primary comorbidity have not been reported on yet. Hence, the aim of the study was to identify reference genes suitable for comparative gene expression analyses using OA-MSCs. MSCs isolated from pelvic bone marrow aspirates of n=13 patients with advanced stage idiopathic hip osteoarthritis and n=15 age-matched healthy donors were used in passage 0. The expression of 31 putative reference genes was analyzed using TaqMan® Express Human Endogenous Control Fast 96-Well Plates (Applied Biosystems). Calculating the coefficient of variance (CV) mRNA expression stability was determined and afterwards validated using geNorm and NormFinder algorithms. Importin 8 (IPO8), TATA box binding protein (TBP), and cancer susceptibility candidate 3 (CASC3) were identified as the most stable reference genes. Notably, commonly used reference genes, e.g. beta-actin (ACTB) and beta-2-microglobulin (B2M) were among the most unstable genes. For gene expression analyses of MSCs from osteoarthritic donors compared to MSCs derived from healthy individuals the combined use of IPO8, TBP, and CASC3 gene is recommended for normalization of qRT-PCR data.
Hypoxia and Mechanical Stimulation Influences Mesenchymal Stem Cell Fate

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Standard conditions in cell culture procedures do not adequately represent the actual in vivo environment experienced by MSC. Numerous factors need to be considered to provide optimal culture conditions that mimic an in vivo situation and test the capability of MSC to survive after transplantation and to function according to the local tissue requirements. In this study we examine the influence of oxygen concentration as well as mechanical stimulation on MSC. Standard oxygen concentration of 21% (normoxia) is widely used for cell culture procedure, even though the oxygen concentration in vivo is much lower, depending on kind of tissue. Cultivation of MSC under hypoxic conditions (5% oxygen) was performed and cell proliferation, metabolic activity and differentiational capacity was examined. Hypoxic conditions caused an increase in the proliferational activity of the UC-MSC. MSC adapted their oxygen consumption and metabolism according to the appropriate hypoxic environment. In order to overcome limitations of long time cultivation, directed differentiation of MSC was also achieved in bioreactors. For guided differentiation towards the osteogenic lineage, MSC were cultivated on ceramic porous 3D matrices under dynamic conditions. Custom-made miniaturized perfusion bioreactors for parallel testing were designed, characterized and optimized for that purpose. The status of differentiation was examined using different histological stainings. The results from this study demonstrated that oxygen concentrations affect many aspects of MSC properties and may be a critical parameter during expansion and differentiation. Mechanical stimulation has also been shown to play an important role in directing MSC fate.
Skin Equivalent Derived from Human TERT Immortalized Keratinocytes and Fibroblasts: Implementation in Wound-Healing Assay

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Currently skin equivalents (SE) used for in vitro assays such as wound-healing make use of primary skin cells. Limitations of primary keratinocytes and fibroblasts include availability of donor skin and donor variation. The use of physiologically relevant cell lines could solve these limitations. The aim was to develop a fully-differentiated SE constructed entirely from human skin cell lines, which could be used as an in vitro wound-healing assay. Skin equivalents were constructed from human TERT immortalized keratinocytes and fibroblasts and compared with native skin and primary SE. Characterization was assessed by immuno-histochemical stainings with markers for epidermal proliferation and differentiation, basement membrane and fibroblasts. Viability was also measured. To test the functionality of the human cell line SE burn and freeze wounds were applied, followed by immuno-histochemical stainings, measurement of re-epithelialization and determination of secreted wound-healing mediators. The human cell line SE was composed of a fully-differentiated epidermis and a fibroblast-populated dermis comparable to native skin and primary SE. The epidermis consisted of proliferating keratinocytes in the basal layer, the spinous layer, granular layer and cornified layer. The human cell line SE was able to re-epithelialize after wounding. Wound-healing mediators were secreted by the human cell line SE after wounding. We succeeded to construct a novel SE entirely from human cell lines. We conclude that this model has the potential to replace currently available constructs based on primary cells. In the future it will be a valuable tool for in vitro wound-healing, e.g. to test therapeutics.
Embryonic stem (ES) cells give rise to the possibility to introduce the stem cell therapy for regenerative medicine, even in the field of drug discovery such as biotherapeutics. There is an urgent need for relevant in vitro assays that make use of ES cells because of their rich biological utility. In this study, we intended to develop in vitro test for efficacy evaluation of gene therapy product. First, we intended to established gene therapy products for this study. We investigated the gene candidates for treatment of ischemic heart disease with the international clinical data and Pub-med. And then VEGF and GATA-4 genes were selected for gene therapy products and DNA plasmid was accepted for Vector system. Thereafter, we induced cardiac differentiation from ES cells with Embryonic Stem Cell Test (EST) method using LIF. Because the rate of functional differentiation of EST method was too high to evaluate for efficacy of gene therapy product on Ischemic Heart Disease, we modified protocol using serial concentration of LIF for relevant cardiac differentiation. We observed that this system revealed VEGF and GATA-4 gene therapy products increased the functional cardiac differentiation and expression of TNNT. These in vitro assay results validated with in vivo Ischemic Heart Disease animal models. Taken together, we studied for establishment of foundation as in vitro test using ES cells for evaluation of ischemic heart disease gene therapy products.
Stem Cell Properties of Rat Fetal Cartilage-derived Cells from Different Developmental Stages

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Fetal cartilage progenitor cells (FCPCs) are a useful source for cell-based therapies for cartilage defect. However, their properties and ability to form tissue as well might vary depending on the developmental stage of donor specimen. This study compared properties of gene expression pattern and multi-lineage differentiation ability in rat FCPCs at different developmental stages of E14, E16 and E20. FCPCs were isolated from condensed mesenchyme (E14), enlarged cartilage (E16), and matured cartilage (E20) of rat fetal limb. Cells were cultured in DMEM with 10% FBS. Mesenchymal stem cells (MSCs) were isolated from rat bone marrow (8 weeks) for comparison. Fetal tissues were stained for chondrogenic markers (Safranin O and collagen type II) and stem cell markers (Sox2). Isolated cells were examined for the expression of collagen type II and Oct4 by immunocytochemistry. Sox2, Oct4, NANOG, collagen type II, Aggrecan, SOX9, RUNX2 were examined using RT-PCR. Multi-lineage differentiation ability was examined. In histological and cytological analysis, the expression of stem cell markers (Sox2, Oct4) became lower, while that of chondrocyte markers (collagen type II, Aggrecan) became higher along with the increase of developmental stages. The similar data presented in gene expression. FCPCs from late stage showed higher multi-lineage differentiation. This study showed that FCPCs from matured cartilage (E20) could improve therapeutic effect of cartilage regeneration by highest chondrogenic markers and differentiation potential. FCPCs from different developmental stage have different properties and these result may important the timing of isolation for cell-based therapies.
Microencapsulation of pancreatic islets within synthetic scaffolds for immuno-isolation purposes is an attractive strategy for the treatment of type1 diabetes. However, major challenges exist to move this technology towards clinical application. In order to address some of the limitations associated with this technology, we have co-encapsulated rat pancreatic islets with rat bone marrow-derived mesenchymal stem cells within biofunctional PEG hydrogel. The synthetic PEG hydrogel scaffold was designed to include glucagon like peptide -1 (GLP-1), RGDS, and IKVAV functionalities. GLP-1 has been used to stimulate insulin secretion function of pancreatic islets, where RGDS and IKVAV peptides have been covalently bonded to PEG hydrogel network in order to provide stem cell adhesion into the synthetic network. The goal is to enhance functionality of pancreatic islets with the aid of physiological peptides such as GLP-1, while immuno-protection from small toxic molecules, such as reactive oxygen species, could be achieved through antiapoptotic properties of stem cells. Individual and peptide combinations were used to explore the synergistic effects of peptide covalent attachment into the hydrogel scaffold. Viability and static incubation assays were used to measure the viability and the amount of insulin release by encapsulated islets. The results demonstrated that coencapsulation of pancreatic islets and stem cells within biofunctional PEG hydrogels are promising, and that viability and insulin secretion of islets could be increased. Encapsulated islets might be protected from immune attack as a result of the presence of stem cells within the close proximity of islets.
A Co-Culture of Adipose-Derived Stem Cells (ASCs) With Different Types of Chondrocyte-like Cells on Porous Membranes

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A co-culture of mesenchymal stem cells and chondrocyte-like cells has been known as one of the effective ways of chondrogenic differentiation. The purpose of this study was to compare cells from different types of cartilage for chondrogenic differentiation of adipose-derived stem cells (ASCs) in the co-culture system. Three different types of tissue have been used in this study: nucleus pulposus (NP), articular cartilage, and enchondroma (EC). In our study, porous membranes were used in the co-culture system for a separate co-culture of ASCs and chondrocyte-like cells while maintaining direct cell contacts. Density of seeded cells was $2.1 \times 10^4$ cells/membrane in co-culture system. After 6 and 10 days of in vitro co-culture of ASCs on membrane, all groups of ASCs showed chondrogenic differentiation as compared with ASCs cultured alone. In particular, we could confirm that the degree of differentiation was enlarged in co-cultured ASCs with enchondroma (EC) through gene expression of collagen type II after 10 days. These results imply that enchondroma cells may be a good cell source for chondrogenic differentiation of ASCs for tissue engineering applications.

Key words: Adipose-derived stem cells, Co-culture, Porous membrane, Enchondroma, Chondrogenic differentiation
Optimization of Culture Conditions for Urine Derived Stem Cell Using Collagen-I and Hypoxic Condition

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Urinary tract derived urine stem cells (USCs) have been proposed as a promising cell source for urological tissue reconstruction. However, the reported culture condition for USCs is hard to achieve the clinical quality and quantity of cell preparation. These drawbacks led us to reconstitute of culture condition by mimic of natural stem cell niche. In this study, we evaluated the effect of combination of extracellular matrix (ECM) proteins and oxygen concentration on ex vivo expansion of USCs. The growth rate, colony forming ability and maintenance of stem cell characteristics of USCs cultured in supplementation of 5 different ECM protein and 2 different oxygen concentrations were analyzed. The results were compared with conventional culture condition. Growth rate was analyzed using CCK-8 analysis. Stem cell marker expressions were evaluated by real time PCR. The growth rate and colony forming ability of USCs cultured in supplementation of collagen-I and 5% O₂ hypoxic condition (USCs\textsuperscript{recon}) was 1.5 and 1.85 fold higher than that of USCs cultured in conventional condition (USCs\textsuperscript{ctrl}). USCs\textsuperscript{recon} showed higher multi-lineage differentiation capacity (neuron, muscle and urothelium) and enhanced the expression of mesenchymal stem cell markers and transcription factors (Nanog, Oct-4 and Sox-2) than USCs\textsuperscript{ctrl}. The chromosomal stability was maintained in both conditions. In conclusion, reconstitution of culture conditions using collagen-I and 5% hypoxia can be an optimal culture condition of uUSCs for clinical application.

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Mesenchymal stem cells have potentials of differentiation into multi-lineages cell types, self-renewal and immunomodulatory effect. The Korean mistletoe lectin (Viscum Album L. var. coloratum agglutinin, VAC) has been known as anti-cancer drug as well as activator for proliferation. However, it is not certain whether VAC affects self-renewal activity of MSCs. Therefore, the objectives are to analyze proliferation of MSCs and expressions of stemness markers (e.g. Oct-4, Nanog, and Sox2). Also, we demonstrated the correlation between self-renewal and IL-6 signaling in MSCs including bone-marrow MSCs (BM-MSCs) and placenta-derived MSCs (PD-MSCs), and normal fibroblast cell line (WI-38) depends on VAC treatment. Self-renewal activities of MSCs were analyzed by MTT assays, qRT-PCR and ELSIA. Low concentration of VAC (5pg/ml) induces to increase the proliferation of MSCs comparing to control (P < 0.05) as well as the expressions of stemness markers (P < 0.05). Interestingly, low concentration of VAC promotes IL-6 signaling in PD-MSCs through alteration of IL-6/STAT3 methylation resulting increasing the self-renewal of PD-MSCs (P < 0.05). Inhibition of IL-6 expression in PD-MSCs using anti-IL-6 antibody induce to decrease their proliferation. Taken together, low concentration of VAC regulates self-renewal activity of MSCs through IL-6/STAT3 signal by changed methylation of IL-6/STAT3. Therefore, these findings suggest that VAC could be useful as an agent capable of controlling limited self-renewal of MSC. Furthermore, it might be helpful data for understanding of the mechanism of self-renewal of MSCs.
Osteoarthritis is the most common musculoskeletal disease in the elderly. Despite recent cell- and tissue engineering advances, repair and regeneration of cartilage defects remains challenging. Induced pluripotent stem (iPS) cells are morphologically very similar to embryonic stem (ES) cells and exhibit high proliferation and differentiation capabilities. Nevertheless, the method to induce chondrocyte-restricted differentiation of reprogrammed cells is particular challenging. Human articular chondrocytes were isolated from osteoarthritic knee joints and reprogrammed by the delivery of defined transcription factors. Successful reprogramming and iPS cell characteristics were examined by gene and protein expression analyses. For chondrogenic differentiation studies, iPS cells were cultivated in a scaffold-free 3-D environment in form of spheroids in presence of chondrogenic differentiation factors such as TGF-β1 and BMP-2 and analysed for the expression of cartilage-specific proteins on cryosections. Generated iPS cell clones expressed numerous pluripotency and ES cell markers, including OCT4, NANOG, SSEA-4, and TRA-1-60 and showed strong alkaline phosphatase staining. These cells were able to form mechanically stable spheroids. The cells in resulting microtissues showed potential to differentiate into a cartilage-like phenotype, synthesizing e.g. proteoglycans. Chondrocyte-derived iPS cells harbour features typical for ES cells and are capable to differentiate into the chondrogenic lineage. Finally, this cell line provides an unlimited cell source for cartilage engineering studies perspective applicable as basis for pharmacological test systems or even for regenerative therapies.
Neural Differentiation of Mouse Embryonic Stem Cells in 3D Culture

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As nervous tissue shows very little capability for self repair following injury, regenerative medicine approaches are increasingly interested in the use of pluripotent cells (such as embryonic stem cells, ESCs) for cell replacement strategies. Many published neural differentiation protocols for ESCs are based on monolayer cultures. However, it is also known that stimulation from the surrounding environment is crucial for the differentiation of cells towards the desired lineage. Biomaterials could be utilized to recapitulate the surrounding 3D physiological environment that cells encounter during \textit{in vivo} differentiation. In order to develop this strategy, we tested whether encapsulation of mESCs within alginate beads could increase neural differentiation with respect to 2D cultures. Alginate was supplemented with the adhesion protein fibronectin (fn), the fn adhesion peptide RGD, and hyaluronic acid (HA, one of the major components of the neural extracellular matrix during development). Cells were cultured following established neural differentiation protocols. In a few days, results showed that the cells were both viable and formed clusters. qRT-PCR and immunocytochemistry analyses demonstrate that cells grown in alginate and alginate-HA show increased differentiation toward neural lineages with respect to the 2D control and to fn and RGD modifications, with higher expression levels of the neural markers BIII-tubulin and NCAM. Immunocytochemistry also reveals that mESCs are able to make connections among themselves inside a cluster but not among clusters. Our data show that alginate and alginate-HA seem to be the best candidates in order to support and enhance mESCs neural differentiation.
Differential Regulation of Cytokine and Growth Factor Profiles Involved In Cell Integration of Collagen and Fibrin Based Scaffolds In Vitro

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Collagen is the base component for the majority of dermal scaffolds. Smart Matrix™ (SM) is a new fibrin based scaffold showing rapid integration and vascularisation in vivo. Wound healing involves both recruitment and differentiation of unspecialised cells and fibroblasts to the site of injury. This is regulated by cytokines and growth factors such as tumor necrosis factor-α (TNFα) (increased in acute and chronic wounds), Transforming Growth Factor β (TGFβ) (increased in scar tissue). Interleukin-8 (IL-8) & Vascular Endothelial Growth Factor (VEGF) (initiates vascular growth in response to injury). The aim of this study was to analyse the cytokine/growth factor expression of placental mesenchymal stem cells (hP-MSCs) and primary human dermal fibroblasts (HDFs) on collagen and fibrin matrices. The expression of released cytokines/growth factors from HDFs (n=3) seeded into contractile collagen gels (CCGs), Integra® (collagen-based), Matriderm® (collagen-based) and SM dermal scaffolds was analysed by ELISA array. Results indicated different expression in Smart Matrix of VEGF (higher), TGF β and TNFα (lower), other cytokines/growth factors were similar between matrixes. To examine the effect of cell substratum on early cytokine/growth factor expression, 5000 passage 4 hP-MSCs (n=3) were seeded onto fibrin (25 µg/ml) or collagen-I (50µg/ml) coated cover slips. 4 hour post-incubation immunocytochemical analysis indicated positive staining for both IL-8 and VEGF on the adhered cells to both fibrin and collagen-I coated samples. These results indicate fibrin based matrices provide a greater angiogenic and reduced fibro-proliferative cell integration response than collagen based matrices.
**Self-renewal and Environmental Plasticity of Neural Crest-derived, Hox-negative Adult Human Chondrocytes**

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In *embryonic* models and *stem-cell* systems, mesenchymal cells derived from the neural crest (‘mesectoderm’) can be distinguished from mesoderm-derived cells by their ‘Hox-negative’ profile, a phenotype associated with a unique capacity of tissue regeneration. Here we demonstrate that also in fully *differentiated* cells from adults, developmental origin and Hox-negativity are associated with a distinct environmental plasticity and self-renewal capacity. Using a hyaline cartilage model, we show that adult human mesectoderm-derived nasal chondrocytes (NC) can be constitutively distinguished from mesoderm-derived articular chondrocytes (AC) by the lack of expression of specific HOX genes (e.g., HOXC4, HOXD8). This HOX-negative molecular fingerprint is associated with multilineage differentiation- and self-renewal capacity at clonal level. In contrast to AC, NC can continuously revert from differentiated to de-differentiated states across serial cycles of cloning, while conserving the ability to generate cartilage tissue *in vitro* and *in vivo* (23% and 60%, respectively). Importantly, NC can adopt the HOX-positive profile of a mesoderm environment upon subcutaneous implantation into a subcutaneous tissue or into articular cartilage defects, where they directly contributed to tissue repair. Our findings identify previously unrecognized regenerative properties of HOX-negative *differentiated* mesectoderm cells in adults and furthermore imply the possible use of NC for the yet open clinical challenge of articular cartilage repair.
Uniform Aqueous Droplets in a Thermal Micro-Fluidic System
Designed for Biopreservation

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Cryopreservation, as the most successful preservation method to date, has been used widely as part of cancer and root cell research to protect and store mammalian cells despite complications. The cell cytoplasm that surrounds the organelles is mostly composed of water causing undesired results during freezing, such as expansion, crystallization, and local increase in solute concentration (solute trapping) due to crystallization. Such damage to the cell can be eliminated by preventing crystallization and vitrifying the cytoplasm by increasing glass transition temperature via introduction of cryo-protectant agent (CPA) carbohydrates such as glycerol in the cell. Exposure to high concentrations of CPAs induces toxic effects and causes damage to the cell due to osmotic stresses and shrinkage. The rate and amount of CPA loading to the cell can be adjusted by controlling the CPA concentration through adjusting the amount of water around the cell. In this study, a new CPA loading protocol enforcing a controlled continuous increase in CPA concentration profile is developed using a thermal micro-channel fluidic system. The new system allows for encapsulation of cells in an aqueous droplet at low CPA concentration and gradual increase of CPA concentration by removal of water from the aqueous droplet into a continuously flowing immiscible organic phase. Soybean oil is employed as the organic phase due to limited solubility of water (0.4-4 % by volume) that is sufficient to achieve the desired aqueous droplet volume by diffusion of water into the thermally controlled oil. As part of the study, a mathematical model that shows the concentration and temperature effects on the system, which also, involves cell effects, is developed.
In several liver diseases (e.g. hepatitis B, C, hepatocarcinomas) there is a visible variation of the organ stiffness. It is known that matrix stiffness increases expression of tumor markers, but studies on the effects of hepatic metabolism or drug induced hepatotoxicity as a function of matrix stiffness are still scarce. As a first step towards establishing meaningful and physiologically relevant in-vitro models of diseased and susceptible liver for the testing of drugs and chemicals, we investigated the relationship between stiffness and hepatic metabolism. In this work we reproduced various grades of stiffness associated with different degrees of hepatic fibrosis using 2D collagen constructs. In particular, collagen was cross-linked with glutaraldehyde so as to obtain surfaces with elastic moduli ranging from 1 to 5 kPa. The constructs were seeded with the human hepatocyte cell line HepG2 and P450 metabolism as well as functional markers such as albumin and urea were assessed as a function of time and substrate modulus. The cells were also assessed using cytoskeletal and immunostaining. Our results show that hepatocytes are highly sensitive to substrate stiffness. Cell proliferation is increased as the substrate gets stiffer and albumin secretion is significantly higher on softer substrates. Furthermore, both exogenous metabolism (CYP1A1, 1A2, 2B, 3A4) and cell morphology are compromised by increasing the degree of collagen cross-linking. Currently drug testing is carried out on healthy young animals, even though they are clearly not predictive of human responses. To this end, further studies will involve the use of 3D constructs in bioreactors to increase the relevance of the model.
Tissue Generation with Chondrocytes on Collagen-Elastin Based Films

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Biodegradable scaffolds are essential tools for tissue regeneration. Collagen and elastin are widely used as they are the main components of extracellular matrix. We have incorporated collagen-elastin in the polymeric construct forming thin films. These components were combined at different ratios and polymerized on tissue culture flasks. Films physical and chemical properties are tested and compared with each other. Human chondrocytes were grown on these films. As revealed by MTT test films had no toxicity and were suitable for cell culturing. Tissue generation tested for extracellular matrix formation. Proper cartilage tissue formation was also observed by histochemical methods.

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Self-Assembled Oligopeptides for Electrochemical Cell Detachment

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We have previously reported cells cultured on a peptide-modified surface can be detached along with desorption of oligopeptide by applying an electrical potential. Here, a new oligopeptide sequences suitable for rapid cell detachment were designed using molecular dynamics calculations and experimental examinations. Molecular dynamics calculations were performed for 80 different combinations of oligopeptides. Oligopeptides consist of three functional domains: the binder domain (C) to bind to a gold surface; the spacer domain (PPP, FFF, etc.) to diminish surface effects on self-assembly; the zwitterionic domain (KEKEKE, KEKE, etc.) to induce self-assembly. Based on the calculations, CPPPKEKEKEKEK, CFFFKEKEKEKEKEK, CFFFKEKEK, and CFKEKEKEK were chosen for experiments. To clarify a key domain and sequence on a dense layer formation, we examined secondary structures of the oligopeptides in bulk water, density of the oligopeptides on a gold surface, and nonspecific protein adsorption onto the modified surfaces. Quartz crystal microbalance measurements revealed that all of the oligopeptides used in experiments forms dense layers on a surface. CD spectra measurements showed that CPPPKEKEKEKEK forms a secondary structure in bulk water while the others do not. Further, water contact angle of the CPPPKEKEKEKEK modified surface was 3 degree, indicating the oligopeptide make the surface super hydrophilic. Furthermore, CPPPKEKEKEKEK modification significantly reduced fibrinogen adsorption compared to those modified with the other oligopeptides or without modification. Based on the results obtained in calculations and experiments, we concluded that CPPPKEKEKEKEK could be the first candidate for the rapid cell detachment.
Umbilical cord tissue is used as natural scaffold in vascular graft implants, wound healing and vocal fold tissue engineering. We have developed a method for generation of neural artificial tissues using human umbilical cord-derived Wharton’s jelly (WJ). In our study we are investigating fresh and cryopreserved mesenchymal stem cells (MSCs) in their natural 3D environment of WJ tissue as possible autologous implants for central nervous system (CNS) therapy in the future. MSCs are directly differentiated towards neural lineages using sequential introduction of growth factors (EGF, bFGF), neurotrophins, small molecules (retinoic acid, dBcAMP) and epigenetic modulators 5-azacytidine, BIX01294, valproic acid and trichostatin A. During the 1st week of differentiation process, WJ MSCs change their morphology, start to express early neuronal marker NF200 and most of the cells are proliferating (Ki67+). Subsequently, MSCs loose characteristic morphology of mesenchymal tissue. MSCs network become more compact with characteristic dense core and the outer region with Ki67+ cells, a prototype of stem cell niche. The cells in mature artificial tissue express also Doublecortin, NeuN and secrete BDNF. Ki67 expression is restricted to the “niche” region. In conclusion we have shown that MSCs residing in natural 3D environment of WJ tissue were responsive to neurogenic stimuli and could be differentiated towards neural lineages, what makes them promising for generation of fully autologous implants for CNS regeneration.
The purpose is to develop a 3D-model of human conjunctiva by tissue engineering, using fibrin-based matrices. A biocompatible matrix, human fibroblasts and epithelial cells from bulbar conjunctiva were used to construct this model. Fibrin matrices were prepared using human plasma or cryoprecipitate. Epithelial cell culture was previously optimized. To characterize cell phenotypes immunocytochemistry was done against E-cadherin, cytokeratin (CK) 7 and 19, MUC5AC, vimentin and ki67 antigens (n=3). Fibroblasts were grown inside the matrices and epithelial cells above them. Hematoxilin/eosin was used to characterize the constructs and AlamarBlue™ assay was used to measure proliferation (n=5). Both fibrin-based matrices allowed cell growth for 14 days. Fibroblasts showed positive reactivity to the stromal marker vimentin, and epithelial cells exhibited positive reactivity to E-cadherin, CK7, and CK19. Some epithelial cells expressed MUC5AC. At day 3 fibroblasts showed better proliferation rates inside plasma matrices than inside cryoprecipitate matrices (1.95 fold increase; p=0.004). On the contrary, epithelial cells showed higher proliferation rates when grown over cryoprecipitate matrices (1.28 fold increase; p=0.022). Constructs seeded with both fibroblasts and epithelial cells, and made from plasma or cryoprecipitate, showed a 3.66 and 2.55 fold increase, respectively, in proliferation rates from day 3 to 7. 3D complete constructs displayed similar consistency to that of human conjunctival tissue. Fibrin-based matrices supported conjunctival cells growth when used either as a scaffold (fibroblasts) or as a substrate (epithelial). It is then possible to bioengineer a fibrin-based 3D model of human conjunctiva.
Controlling 3D Configuration by the Creation of Cell laden Scaffolds for Hepatic Tissue Engineering

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Since cellular cross-talk can be essential for the maintenance of hepatocyte functionality, we aimed to build a model that allows controlled co-culture configuration in a three dimensional cultivation system. Our first goal was to find a suitable matrix for the cultivation of HepG2 cells, which serve as model for hepatocytes. The cells were encapsulated in gelatin hydrogel discs and different encapsulation parameters were varied to optimize cell viability up to 91%. To meet the functional requirements, the gelatin hydrogel was further modified to contain galactose. Cultivation in the galactosylated hydrogel improved the expression of hepatocyte specific genes such as albumin, TTR and HNF4α without affecting cell viability. Our second goal was to control the 3D configuration of this gelatin-cell construct by the creation of a scaffold with pre-defined dimensions. Scaffolds were created using the Bioscaffolder technology and different parameters were varied to optimize cell viability. Currently we are able to bioplot cell-gelatin scaffold with a comparable cell viability (93%) as in the previous mentioned discs. The proposed material is suitable for the 3D cultivation of HepG2 cells and further modification with galactose improved the expression of hepatocyte specific genes. The 3D configuration of this cell-gelatin construct can be controlled by using the Bioscaffolder technology. Further investigation will show if co-culture with other cell types is possible on these scaffolds and how this will influence hepatocyte specific functions.
Identification of Laminin Expression During the In Vitro Development of Bioengineered Human Cornea, Skin and Oral Mucosa

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Generation of functional epithelial-stromal artificial tissues is strongly dependent on the development of a mature basement membrane. Laminin modulates epithelial cell adhesion, differentiation and migration, and it is the most abundant structural and biologically active component of the epithelium-mesenchyme adhesion system. The aim of the present work was to analyze the immunohistochemical expression of laminin in bioengineered human cornea, skin and oral mucosa substitutes. Primary cell cultures of cornea, skin and oral mucosa epithelial cells and fibroblasts were obtained from human tissue biopsies. Then, fibrin-agarose stromal substitutes were generated with cultured fibroblasts immersed within, and the epithelial cells seeded on top. H&E staining and immunohistochemical analysis of laminin were performed after 1, 2 and 3 weeks of development in culture. Histological evaluation of the cornea, skin and oral mucosa constructs revealed the progressive development of a multilayered epithelium and the presence of an integrated and well-formed artificial stroma. Interestingly, an orthotypical pattern of laminin expression was identified in the artificial cornea model after 1 week of development, and after 3 weeks in the skin and oral mucosa bioengineered models. These results suggest that fibrin-agarose artificial stromas allow the efficient development and maturation of a basement membrane in different complex tissues such as the human cornea, skin and oral mucosa.

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Low generative potential of the nerve tissue causes high level of lethality and disability this is why research on the development of advanced matrixes to support the migration/growth of the nerve cells and promote local angiogenesis is an eminent task. In our study the gelatin matrixes produced by cryogelation (gelatin cryogels, GC) were tested as potential biomimetic material for the replacement of autologous nerve grafts. For in vitro testing the primary cells isolated from spinal ganglia of CD1 mice were used. The co-culture of ganglion neurons, glial cells and fibroblasts has been used for the observation of outgrowth of neuritis in neurons and proliferation of glial cells and fibroblasts on polyornithine and laminin modified GC. Preliminary, grows of the cells was analysed on the gelatin, polyornithine and laminin modified polystyrene surface. Polyornithine and gelatin–polyornithine films were found to support the attachment and survival of ganglion cells on polystyrene. While polyornithine and gelatin–polyornithine modified surfaces did not efficiently promote neurite outgrowth in neurons the additional adsorption of laminin onto polyornithine and gelatin–polyornithine films resulted in the enhanced neurite outgrowth. Laminin also markedly stimulated the proliferation of glial cells morphologically resembling Schwann cells. The modification of GCs with polyornithine significantly promotes the proliferation of glial cells in the matrix. Modification with laminin further increased the number of glial cells in the GC. The results could form the ground for the further development of modified GCs as conduits for the peripheral nerve regeneration. The work was supported by PERG08-GA-2010-276954 and Russian Federal Programme grant № 14.A18.21.1236.
Bone inducing proteins like BMP’s are in commercial products combined with collagen based carriers of bovine origin that causes an immunogenic reaction. For such bone graft substitutes the biocompatibility and non-immunogenicity of the material are crucial. It is therefore important to develop new carriers. A promising alternative may be the use of injectable carriers based on biopolymeric compounds. We examined the in vivo tissue response of preformed hyaluronan-based hydrogel implanted subcutaneously in rats by staining with monoclonal antibodies and flow cytometric analysis. The tissue response was followed in series of experiments at 1, 4, 7, 14, and 28 days, to include both the immediate and the long-term tissue response to the hyaluronan-based hydrogel. The hydrogel explants were degraded and the cells were analyzed by flow cytometry to quantify cell types. There were no cases of cutaneous reactions. Distinct populations of cells expressing the leukocyte common antigen CD45 and ED2-like antigen for macrophages were present. Granulocytes were present, but not in distinct populations. A small number of CD31+ cells (endothelial cells) were also present. The results showed cell ingrowth after 4 days and the presence of cells increased over time; meanwhile the percentage of immune cells decreased, especially the CD45+ population (leukocytes). The CD31+ population remained stable. The migration of cells increased over time, although the percentage of immune cells was low and decreased over time. These results indicate that the hyaluronan-based hydrogel displayed good in vivo biocompatibility.
Adipose-Derived Stem Cells: Isolation within the Intraoperative Timeframe and Characterisation

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The use of adipose-derived stem cells (ASCs) as an autologous and self-replenishing source of tissue provides much promise in reconstructive surgery. Multiple methods of extraction of pluripotent adipose stem cells from lipoaspirated tissue have been described. The aim of this research was application of novel time- and yield- efficient ASC isolation protocol, which can be applied for use in the intraoperative timeframe to ameliorate results in reconstructive surgery. Six patients undergoing free fat transfer procedures donated surplus adipose tissue collected by the Coleman method from the abdomen for isolation and characterisation of ASCs. 10 grams of adipose tissue were washed, digested, centrifuged and filtered to obtain the ASCs pellet, which was cultured for 7 days. Cells were then FACS characterised using cell markers CD14, CD45, CD73, CD90 and CD105 and HLA-DR. Cells were isolated using a time-efficient protocol totalling two hours 30 minutes. Cultured cells largely stained positive for CD73, CD90 and CD105, as expected from ASCs, and negative for CD14 and CD45. An ASC isolation protocol suitable for use within the intraoperative timeframe has been identified. This may be further time-constrained from 2 hours 30 minutes to under one hour, by reduction of digest time of adipose tissue. Further manipulation of ASCs is advisable by directed differentiation, to demonstrate their multipotency.
Origin of Pericyte-like Cells in Co-cultures of Mesenchymal Stem Cells and Endothelial Progenitor Cells

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In tissue engineering approaches for large bone defects it is important to promote vascular ingrowth into the constructs to ensure a sufficient supply of oxygen and nutrients. Perivascular cells contribute to the stability and maturation of micro-vessels. Our previous work has identified a pericyte-like cell population as part of the newly formed capillary-like structure following the co-culture of human bone marrow-derived mesenchymal stem cells (MSCs) and Endothelial Progenitor Cells (EPCs) within scaffolds. In this study we aimed to determine the origin of this cell population and the stimulus of their induction. We obtained mononuclear cells and EPCs from human bone marrow using Ficoll and CD34+/CD133+ MACS® separation, respectively. MSCs and EPCs were seeded in a) indirect co-culture (paracrine interaction), b) in direct co-culture and c) in single cultures. Cells were cultured for 10 days in IMDM-10%FCS or IMDM-5%FCS-5% Platelet Lysates (PL) medium. Gene expression of pericyte markers CD146, NG², αSMA, PDGFRB were analyzed using RT-PCR at day 0, 3, 7 and 10. Comparing MSCs in the three conditions we observed an increase in the gene expression of the four markers in direct co-culture. These in-vitro preliminary results suggest that pericyte-like cells derive from MSCs and that direct cell to cell contact with EPCs is an important event for this differentiation process to occur.
Bioactive Nanofibers Functionalized with Anti-CD4 Antibody for Improved Cell-based Therapy

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Immune cell-based therapy has attracted a great deal of attention for the treatment of cancer in current clinical studies. In particular, adoptive T cell therapy has been widely used particularly due to the potential to enhance immune responses. However, commonly performed anti-cancer therapy has several limitations; activity of cells is often significantly suppressed when they are cultured in vitro conditions and the transplanted cells are often difficult to be retained in appropriate target places for long-term activity. To address these problems, we developed a surface modification technique of immobilization of anti-CD4 antibody to PLGA nanofibers. After the fabrication of nanofibers functionalized with antibodies, we measured isolation efficiency of CD4 T cells and cultured immune cells on the nanofibers to observe their proliferation and release. In addition, we found that the T cell isolation efficiency was significantly increased when the fibers were exposed to spleenocytes containing multiple cell types such as B-cells, T-cells, and NK cells. Collectively, our results suggest that surface immobilization with anti CD4-antibody on the biocompatible and biodegradable nanofibers can be a powerful method to isolate, to stimulate the proliferation of and to release CD4 T cells from the nanofibers. Therefore, our system can present a new therapeutic platform to improve CD4 T cell based immune responses and can be used as a novel delivery system of anti-cancer CD4 T cells.

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Supercooling as a Viable Non-freezing Cell Preservation Method of Rat Hepatocytes

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Supercooling preservation holds the potential to drastically extend the preservation time of organs, tissues and engineered tissue products, and fragile cell types that do not lend themselves well to cryopreservation or vitrification. Here, we investigate the effects of supercooling preservation (SCP at -4 °C) on primary rat hepatocytes stored in cryovials and compare its success (high viability and good functional characteristics) to that of static cold storage (CS at +4 °C) and cryopreservation. We consider two prominent preservation solutions a) Hypothermosol (HTS-FRS) and b) University of Wisconsin Solution (UW) and a range of preservation temperatures (-4 to -10 °C). We find that there exists an optimum temperature (-4 °C) for SCP of rat hepatocytes which yields the highest viability; at this temperature HTS-FRS significantly outperforms UW solution in terms of viability and functional characteristics (secretions and enzymatic activity in suspension and plate culture). With the HTS-FRS solution we show that the cells can be stored for up to a week with high viability (~56 %); moreover we also show that the preservation can be performed in large batches (50 million cells) with equal or better viability and no loss of functionality as compared to smaller batches (1.5 million cells) performed in cryovials.
3D Culture and Differentiation of Human Mesenchymal Stem Cells towards Nucleus Pulposus in a Physiologically Informed Bioreactor

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The Intervertebral disc (IVD) has a key role in compression modulation. The nucleus pulposus (NP) is the central, gelatinous, core of the IVD and can experience high compressive forces resulting in loss of structural proteoglycans and increased expression of matrix degrading proteins. Ultimately this can lead to disc degeneration and subsequent chronic back pain. Current, clinical treatments alleviate pain for short-term periods but are highly invasive; therefore tissue engineering and stem cell therapy may provide a more effective treatment. Here, human mesenchymal stem cells (hMSCs) were encapsulated into a synthetic polymer hydrogel (PHEMA-co-APMA grafted with PAA) and exposed to accepted levels of UV. hMSC-gel constructs were cultured in hMSC media and chondrogenic media up to day 21 in reduced oxygen conditions and cultured in both static and mechanically stimulated (axial compression) conditions. Results demonstrated cell viability of cells encapsulated within hydrogels and increased cell numbers from day 0 to day 21, in both static and mechanically stimulated conditions. Cells were characterised for hMSC marker expression and protein expression levels for NP markers (collagen and aggrecan) in the various conditions (media and mechanical stimulation) investigated. Additionally, mechanical properties and degradation behaviour of acellular and cellular hydrogels was also monitored. Greater degradation was observed in stimulated, acellular hydrogels versus static conditions. This injectable hydrogel supported successful hMSC encapsulation and differentiation towards NP cells demonstrating potential use for clinical therapies to restore the function and properties of NP tissue.
Tissue engineering is based on the use of various biomaterials either cell-free or in combination with specialized cells for regeneration of damaged or destroyed tissue. For cartilage regeneration biomaterials have to feature good biocompatibility and biodegradability, allow proper cell adhesion and promote the establishment and stabilization of a chondrocytic phenotype of cells. In this study we examined the effects of a porous 3D scaffold of cross-linked hyaluronic acid on the gene expression and synthesis performance of chondrocytes. Therefor human osteoarthritic chondrocytes were expanded and seeded onto the scaffold. This was followed by a 3 week cultivation period. For estimation of the cell content, metabolic activity and DNA-content within the scaffold were determined. The expression of chondrocyte-specific genes as well as the synthesis of sulphated glycosaminoglycans (sGAGs) by embedded cells was analyzed to characterize the synthesis performance of the cells. Within the scaffold cells showed a homogenous distribution. Although DNA-quantification indicated only partial loss of cells, while the metabolic activity within the scaffolds decreased dramatically during cultivation. This might be attributed to a stop in cell proliferation combined with a switch of the cellular genetic program from cell division to differentiation towards a chondrogenic phenotype. Gene expression analyses of chondrogenic markers and analysis of sGAG synthesis substantiated this hypothesis as both chondrocyte specific gene expression as well as sGAG synthesis were increased and the differentiation index was clearly improved. These results suggest that the investigated material has a chondroinductive effect on embedded cells.
Biomimetic Peptide Nanofibers Support Cardioprogenitor Cells for Cardiomyocyte Regeneration

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Myocardial infarction is a leading cause of mortality worldwide, since adult mammalian cardiomyocytes have limited proliferation capacity for renewal after ischemia caused by infarction. Regenerative medicine that aims to enhance cell population in the damaged area and improve tissue function is a promising approach for treatment of ischemia. Mimicking extracellular matrix (ECM) using various scaffolds (such as natural and synthetic polymers) is an effective method for regenerative medicine studies and peptide amphiphiles (PA) molecules are versatile materials that can self-assemble into bioactive, biodegradable hydrogel scaffolds. Heparin is a highly sulfated glycosaminoglycan that resides in ECM and organizes cellular behaviors by interacting with growth factors. In this study, a heparin mimetic PA (GAG-PA) was utilized as scaffold materials that can be used for cardiac regeneration. After chemical, physical and mechanical characterizations of molecules, in vitro studies were carried out to investigate behaviors of cardiac muscle progenitor cells on PA gels. Viability, proliferation and adhesion of cells were assessed to determine cell-matrix interactions. Subsequently, differentiation of cardiac progenitor cells was investigated by immunocytochemistry and qRT-PCR analysis. Our results showed that these bioactive PA hydrogels can induce proliferation of cardioprogenitor cells and cardiomyocyte differentiation.
Maturation of Newly Deposited Calcium-Phosphate Crystals in Human Adipose Derived Stem Cells after their Differentiation to Osteoblast in vitro

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In vitro mineralization is a good indicator of osteoblasts maturity and functionality. Numerous techniques are used to detect mineral deposits but amount of data characterizing mineral composition are modest. The aim of the study was to better understand the maturation of mineral formed in vitro. We compared mineral formation, maturation and composition of human ASC (Adipose derived Stem Cells), BMSC (Bone Marrow Mesenchymal Stem Cells) after incubation in osteogenic medium to bone apatite. ASC and BMSC were cultured in standard osteogenic medium (OM) containing DMEM, FBS, dexamethasone, ascorbic acid-2-phosphate and one out of two phosphate sources: β-glycerophosphate or NaH2PO4. Deposited mineral was intended for subsequent analysis on days 17, 21, 24, 28, 35, 42 and 51 of the experiments. Alizarin Red staining, Transmission Electron Microscopy examination, quantitative analysis on Electron Paramagnetic Resonance, Fourier Transform Infrared Spectroscopy and X-ray Diffraction was performed. Results obtained from each method revealed differences in ASC and BMSC mineral deposition rate and its maturation in in vitro culture. We found that mineral from earlier time-points during in vitro differentiation was in form of amorphous apatite that gradually at later time-points transformed into the mature crystalline apatite mineral. Both forms of apatite were stained with Alizarin Red. Summarizing, Alizarin Red staining does not distinguish between immature and mature mineral what may lead to false conclusions. Only battery of different methods used in the study allowed us to detect and monitor mineral maturation.

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Controlling Spatial Heterogeneity in Cell Density in Collagen Constructs

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A key requirement of any scaffold is the ability to promote cell function – the material must support cells within a 3D environment that mimics the architecture, biomechanical and biochemical environment of in vivo tissue. One option is to seed cells in a collagen gel – plastic compression increases the collagen density (and hence mechanical integrity) to representative in vivo values. This approach has been shown to sustain oxygen concentrations at physiological values throughout a construct. An oxygen gradient is established radially through the construct, as a consequence of consumption by the cell population. This results in heterogeneity in cellular proliferation and death rates, as well as chemotactic migration. The current study investigates cell fate spatially in such a construct, using a mathematical model that tracks the concentration of oxygen, and density of cells, fluid and collagen throughout the construct. Constitutive laws are proposed to describe the stresses within each phase (cells, fluid or collagen), interactions between phases (e.g. drag between the cells and scaffold), and cell processes (such as cellular aggregation, contact inhibition and chemotaxis). The models are parameterised against time course data on measured oxygen concentrations and cell viability at different spatial positions within the construct. The model is used to predict optimal seeding distributions and construct dimensions that ensure culture of a functional and controlled cell population.
Placenta has several functions on behalf of healthy embryonic development, besides a good source of stem cell. Both amniotic and chorionic mesenchymal stem cells are isolated from term placentas. Sox2 plays key role in maintaining self-renewal, or pluripotency of undifferentiated stem cells. Oct4 is also used as a marker for undifferentiated cells. It is reported that both amnionic and chorionic mesenchymal stromal cells express Oct4, Sox2 mRNAs in human term placenta. Diabetes mellitus is a metabolic disorder that affects millions of people worldwide. In diabetic pregnancies several placental disorders may adversely affect intrauterine life. Therefore we wanted to test whether abnormal placental growth in diabetic patients will be associated with placental stem cells or not. We analyzed Oct4 and Sox2 expression in human term placentas by Western Blot and Immunohistochemistry. We found that Oct4 and Sox2 expression was positive at human term placenta. Oct4 protein level decreased in diabetic placentas but Sox2 level was similar. According to our immunohistochemistry results, Oct4 and Sox2 were positively stained in chorionic mesenchymal stromal cells. Immune reaction densities were similar between the groups. Interestingly, Oct4 and Sox2 were also positive at syncytiotrophoblasts. Although these proteins are pluripotency markers, Oct4 and Sox2 might have different roles in different tissues and cells. Pathologies in diabetic placentas will be related with abnormal cellular proliferation/differentiation mechanisms including placental stem cells. It is worth to study the pluripotency of placental stem cells in pathological conditions in detail, because it could be the main reason lying underneath the abnormal processes.
Galectin-3 and Collagen IV Rescue Tubular-like Structure Formation Under Dynamic Conditions In a Bioreactor System

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Nutrient and oxygen supply are essential for an engineered tissue with more than 100-200µm of thickness, which can be ensured by blood vessels. However, formation of tubular-like structures in a co-culture system of human foreskin fibroblasts (HFFs) and human umbilical vein endothelial cells (HUVECs) under dynamic conditions has not been investigated yet. Additionally, the effect of galectin-3 and collagen IV in this system exposed to flow is examined. The HFF/HUVEC co-culture system has been moulded in fibrin gel and cultivated for 9-21 days under static conditions. In comparison, 14 days of dynamic co-culture was performed using a custom-made bioreactor system. Furthermore, the effect of galectin-3 (10µg/ml) and collagen IV (30µg/ml) on the formation of tubular-like structures has been investigated. After 9-21 days of cultivation, gels were fixed and immunostained (CD31). Visualization of tubular-like structures in the 3D fibrin matrix was carried out using two-photon microscopy and ImagePro® Analyzer software.

The HFF/HUVEC co-culture system under dynamic conditions led to less tubular-like structures compared to static conditions. Addition of galectin-3 and collagen IV rescued the tubular-like structure formation under dynamic conditions.

Tubular-like structure formation using the HFF/HUVEC co-culture system can be realised under dynamic conditions with the help of galectin-3 and collagen IV. With regard to other co-culture systems exposed to flow, a strong stimulus seems to be required to obtain tubular-like structures.
Single Enzyme Isolation and Characterization of Human Placenta Mesenchymal Stem Cells

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Human placenta is a readily available, highly vascular tissue with abundant sources of mesenchymal stem cells (MSC). The aim of this study was the isolation and characterisation of human placenta derived MSC for use in wound repair and identification of the expression pattern of stem cell markers Stro-1, CD29, CD34, CD44, CD90, panCD45, CD73, CD104, CD133, CD166, CD177, and previously unreported Sox-2 (SRY-related HMG-box (SOX)) & CD18 (Integrin β-2). Human term placentae were kindly donated by Watford general hospital (n=3). Isolated placental cotyledons, were finely minced and sieved through a 250 µm metal sieve to collect tissue fragments. Digestion with Collagenase IV (3000 U/ml) was performed for 45 minutes. The digested cell pellet was then centrifuged and resuspended in MCDB 131 media and then disaggregated through a needle. The cell suspension was plated onto gelatin (1%) coated culture plate. MCDB 131 media containing 50U/ml Penicillin, 1 µg/ml Hydrocortisone, 50 µm Dibutyryl cyclic adenosine monophosphate, 5ng/ml EGF and 20% human heat inactivated serum media was then added and cells were incubated at 37 °C, CO2 incubator. Collagenase IV only digest resulted in cell sprouting as early as day 3 and light microscopy indicated confluent culture wells of spindle like cells by day 14. Flow cytometry and Immunocytochemistry results were positive for Stro-1, Sox-2, CD133, CD 166, CD104, CD177, panCD45, CD90 and CD44 and negative for CD34 and CD18 suggesting an MSC phenotype. Osteogenic, adipogenic and chondrogenic differentiation of the isolated cells confirmed their stromal nature. HP-MSCs can be used in conjunction with biomaterials to study mechanisms of tissue repair, and have potential for tissue regeneration.
Advances in Representation and Visualization of Microarray Analysis of hASCs for Tissue Engineering Applications

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Microarray expression studies are generating large volumes of data, which should be deeply analysed to obtain their significance. As more quantitative data are available, as much is increasing the necessity of data mining techniques for dealing with these huge databases. Our main objective was to apply new Bioconductor tools for the visualization of the results obtained through expression microarrays for helping basic researchers that are not familiar with Bioinformatics to interpretate their results. Microarray studies were performed with human adipose stem cells (hASCs). Different cell populations were isolated by size from samples using FACs and their gene expression profile was studied using gene expression microarrays. Differential expression analysis was performed using R v2.15.1 and Bioconductor v 2.12 statistical packages. Bioconductor is a collection of R packages for the analysis and comprehension of high-throughput genomic data. Since 10 years, this collection has growth to more than 600 packages for many domains, especially for expression analysis. Background correction was applied by the subtraction method described in LIMMA. Array data were normalized through a non-parametric quantile normalization assuming similar distribution of the signal for all arrays. Our observations suggest that novel contribution in Bioconductor statistical packages can yield better interpretation different results depending on sample size and functional genes of interest. With this tools we have characterize after their isolation from hASC, two subpopulations of cells that express early developmental markers. All the candidates obtained were validated by PCR.
Clinically Competent Human Embryonic Stem Cell Derived Vascular Cells form Vascular Networks

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Adult endothelial progenitor cells derived from various sources have been reported for therapeutic angiogenesis. However, obtaining sufficient number of functional and transplantable cells is a critical challenge. In this regard, human embryonic stem cells (hESCs) due to their ability for exponential growth and differentiation to various lineages could be used as an unlimited source to generate huge amounts of functional and transplantable vascular cells. However, current strategies to differentiate hESCs to vascular cells are limited by inefficiency and use of xenogenic products. We aim to differentiate hESCs to endothelial cells (hESC-ECs) and vascular smooth muscle cells (hESC-vSMCs) with minimal use of xenogenic products. H1 hESCs were differentiated to vascular progenitors using a novel protocol in chemically defined, animal component-free conditions. Differentiation of hESCs to vascular progenitors was induced by sequential modulation of Wnt-βCatEnin (inhibition of glycogen synthase kinase-3), FGF and VEGF pathways. These vascular progenitors were further differentiated to hESC-ECs and hESC-vSMCs in serum-free conditions and extensively characterized. When these vascular cells were embedded within Polyethylene Glycol-Fibrin gels they formed anastomosing cord-like vascular networks. In conclusion, our findings demonstrate the potential to efficiently derive clinically competent hESC-ECs and hESC-vSMCs with minimal use of xenogenic products. Further, these vascular cells could be used to fabricate in-vitro vascularized constructs for various tissue engineering purposes.
Upregulation of RANKL in Bone Metastatic Breast Cancer Cells after Direct Co-Culture with Osteogenically Differentiated Human MSCs

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Bone metastases arise in nearly 70 percent of patients with advanced breast cancer, leading to massive bone lysis. RANKL/RANK/OPG pathway is the key molecular axis for osteoclasts formation, regulating both normal bone resorption and metastatic bone lysis. This work aims at investigating the reciprocal interactions between fluorescent human bone metastatic breast cancer cells (BOKL) and bone-derived cells (MSCs). MSCs were harvested from 3 different donors, cultured for 14 days in osteogenic medium and tested for differentiation. BOKL were cultured in growth medium (CTR), in direct co-culture and in medium conditioned by the same MSCs (CM). After enzymatic detachment and FACS sorting of the two cell types, real time PCR for proliferation and migration related genes was performed. Alizarin red staining and assay for calcium content confirmed osteogenic differentiation of MSCs. PCR demonstrated RANKL up-regulation up to 17 fold in BOKL in direct co-culture with MSCs as compared to CTR. On the contrary, we did non observe significant up or down regulation for genes of BOKL cultured in CM. Furthermore, RANKL decoy receptor OPG was 2-fold up-regulated in BOKL directly co-cultured with MSCs from each of the 3 patients. In conclusion, through gene expression analysis in accurately separated cell populations following direct co-culture, we reliably showed that direct but not indirect co-culture between BOKL and bone differentiated MSCs increased expression of key genes in metastatic cells. Thus demonstrating the fundamental role of direct contact between bone metastatic breast cancer cells and bone cells in the initiation of the vicious cycle causing bone resorption and consequent metastatic cells growth.
Comparison of Basis mRNA Levels of Tendon Markers in Multipotent Mesenchymal Stromal Cells Derived from Different Sources

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Tendinopathies are among the most often seen disorders in orthopedic patients. Due to the poor healing capacity of tendon tissue following conservative and surgical treatment, new therapeutic approaches have moved into the centre of attention. A promising option is the intralesional application of multipotent mesenchymal stromal cells (MSCs), which is already in clinical use in equine medicine. Several studies proved that differences exist between the cell characteristics of MSCs derived from different sources. However, variations in tendon regeneration potential have not been studied yet. MSCs from different equine tissues (bone marrow, adipose and tendon tissue, umbilical cord blood and matrix) were isolated and characterized. Subsequently, the obtained MSCs were used for gene expression analysis of tendon markers by real time reverse transcription polymerase chain reaction (RT-PCR). Genes of interest were the commonly used tendon markers collagen 1, collagen 3, scleraxis, decorin, tenascin C and tenomodulin. MSCs derived from adipose tissue showed the highest expression of the extracellular matrix components collagen 1, collagen 3 and decorin (p<0.05). MSCs derived from umbilical cord blood showed expression of the tendon related genes scleraxis and tenascin C at a high level. Tenomodulin, which has been described as a late tendon marker, could not be detected by real time RT-PCR.

In conclusion, the origin of MSCs has an impact on their basic expression of tendon markers. MSCs derived from adipose tissue and umbilical cord blood might be most suitable for use in tendon therapy.
Differences in Rigidity Sensing by Human Adipose Derived Stem Cells in the First Days of In Vitro Culture

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The influence of the stiffness of cell support on stem cell differentiation is intensively exploited research area. Osteogenic differentiation of mesenchymal stem cells is often evaluated after long periods of in vitro cultures, when the mineralisation can be observed. However cells react to the physical properties of the support from the beginning of the culture. In this work we analyzed the response of human adipose derived stem cells (hASCs) toward substrate elasticity in the first days of culture with particular attention paid to osteogenic differentiation. In vitro cultures of hASCs on substrates of tissue-like stiffness were observed on days 0, 4, 7, 14, 21. Collagen I coated inert polyacrylamide gels of 2 different rigidities (1.6kPa, 28.1kPa) were used. Cytoskeleton and focal adhesion were evaluated by β-actin and vinculin staining. Cell viability (XTT) and cell number (PicoGreen) were assessed. Differentiation potential was determined by measuring ALP activity and expression of ALP and RUNX2, as osteogenic differentiation markers (real-time PCR). The support had no influence on cell number or viability, but the morphology was clearly affected by the rigidity. hASCs differentiation confirmed by RUNX2 and ALP expression was detected in all groups. RUNX2 and ALP expression was higher (2 fold) on more rigid substrates as compared to the softer ones, both on day 4 and 7. This was accompanied by the enhanced ALP activity on day 7 (1.5 fold). There were no differences in RUNX2 and ALP expression as well as in ALP activity on day 14 and 21. We propose that hASCs react to the rigidity of the support in early time points, when the cell-substrate interaction is not affected by other cells or high amount of produced ECM.

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Generating a Neuronal Network In Vitro Model – Towards Therapies for Huntington’s Disease

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Huntington’s disease (HD) is a neurodegenerative disease that damages circuitry in the brain responsible for regulating movement. An in vitro model, based on the striatum (the area mostly affected by HD), could provide essential data on optimal conditions required to re-establish circuitry after damage and provide a route to potential therapies for HD. The first step of designing this HD model involves controlling neuronal adherence and neurite orientation in vitro. This can be achieved by culturing neurons on aligned topography, i.e. nano-fibers and micro-grooved substrates made via electrospinning and soft lithography, respectively. Poly (lactic acid) (PLA) fibers were either attached to coverslips or incorporated into collagen gel constructs. Polydimethylsiloxane (PDMS) was cast over inverse templates such that micro-grooved substrates were formed. Dissociated primary rat neurons (E16) were cultured on these substrates. Prior to cell culture substrates were either pre-coated with poly-L-lysine (PLL) and laminin or pre-seeded with astrocytes. Poly-L-lysine and laminin provided the best chemical cues for attachment of CNS dissociated neurons. Once attached, neurites responded to surface topography, aligning with directional guidance. Astrocytes were much more responsive to aligned nanofibres compared to neurons. Co-culture of neurons on pre-seeded astrocytes gave rise to improved neuronal viability, although neural alignment was only slightly improved. In general, the substrate topography was the major influence over neurite orientation compared to biological (astrocyte) cues. Continuing work involves the use of a microfluidic device to form a neuronal network towards mimicking the circuitry damaged by HD.
Improvement of Differentiation Efficiency into Insulin-Secreting Cells by Ngn3 and Pax4 Expression

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For long-term treatment of diabetes type 1, transplantation of the insulin-producing beta cells might be a promising method, but the limited number of islets for transplantation conducts to develop different approaches. In this project, Pax4 and Ngn3 genes isolated from rat pancreatic islets were transferred into pancreatic islet derived stem cells, and the effect of this ectopic expression on the differentiation efficiency was examined. By pancreatic duct ligation, the tissue damage on Wistar albino rats was developed to increase the expression of Pax4, and Ngn3. These genes were cloned into the appropriate expression vectors and transferred into stem cells by electroporation. After stable ectopic expression of Pax4 and Ngn3 genes were obtained, the insulin synthesis levels of these cells cultured in beta-cell differentiation media (betacellulin, HGF, nicotinamide, B27, b-MEtoH) were analyzed. After isolation, Pax4 and Ngn3 genes were obtained in length of 645bp and 1050bp, respectively. Their expression was achieved in cells by cloning into pFLAG-4 expression vector (Sigma). Following the differentiation step, insulin-secreting cells were attained, and the expression significantly increased their rate of insulin secretion. The altered insulin secretion levels were detected in response to different glucose levels. Conclusively, Pax4 and Ngn3 ectopic expressions in the cells result in improved differentiation efficiency into insulin secreting cells.
The Role of Myeloid Cells in The Neovascularization of Bone Tissue

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According to the present knowledge mixed populations of blood derived endothelial progenitor cells (EPC) might act as proangiogenic myeloid cells, which play a significant role in the regulation of angiogenesis and blood vessel reorganization. In this context, we have evaluated the contribution of blood derived EPC to the formation of vascular structures in an in vitro model for bone repair consisting of human mesenchymal stem cells and human blood derived outgrowth endothelial cells (OEC) and evaluated the molecular effects. OEC and EPC were isolated and characterized according to previously published protocols from the peripheral blood. MSC were harvested from bone as described before. Co-cultures were set up by seeding MSC first followed by the addition of OEC in a ratio of 1:1. Then we added 5% or 10% EPCs per total cells to the system. MSC with 10% EPC and monocultures of MSC or OEC were used as control groups. Vascular structures were analyzed by CLSM for endothelial and myeloid markers and quantitative image analysis. The molecular effects of myeloid cells on the systems were evaluated by quantitative real time PCR, ELISA, and protein arrays from cell culture supernatants and lysates. The addition of EPC results in a significant increase in the signal for myeloid cells but also in the increase of endothelial markers indicating beneficial effects on endothelial cells growth. The formation of prevascular structures was significantly improved by the addition of EPC. We identified several candidate molecules involved in the regulation of blood vessel formation and the attraction of myeloid cells to sides of neovascularization. In addition, the expression of CD 68 indicates that EPC at least partly act as macrophage like-cells.
Complex 3D Architectures using a Textile Technology for Bone Tissue Engineering Applications

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Textile-based technologies are particularly interesting in tissue engineering since they allow producing finely tuned fibre-based porous structures, offering superior control over the material design (size, porosity, fibre alignment) and manufacturing. Scaffolds with very reproducible and interconnected intra-architectural geometry can be processed increasing the surface area for cell attachment and tissue ingrowth. This work aims to evaluate the potential of recently developed 3D textile structures based on silk fibroin (SF) to support human Adipose-derived Stem Cells (hASCs) adhesion, proliferation and osteogenic differentiation. These cells constitute an emerging possibility for regenerative medicine, including for bone tissue regeneration. A comparative analysis was performed with a more stable polymeric system, polyethylene terephthalate (PET). SF and PET yarns were processed into 3D spacer structures using warp-knitting technology. The obtained complex 3D architectures are composed of two knitted layers assembled/spaced by a PET monofilament to increase the tri-dimensionality of the scaffold. Cells were able to attach to the fibres, proliferate and differentiate into the osteogenic lineage. hASCs were able to deeply penetrate into the scaffold and colonize its interior with great evidences of extracellular matrix mineralization (Fig.1). The efficiency and high level of control of the warp-knitting technology together with the interesting structural properties of the resulting constructs makes this a very versatile and adaptable system to the specific bone tissue anatomy and function.

Fig.1- SEM micrograph showing hASCs penetration and attachment to the fibres of the complex 3D spacer architectures.
Schwann cells are a crucial factor in peripheral nerve regeneration. As new approaches are sought, there is an increasing demand of native Schwann cells for in vitro testing and/or reimplantation. In this study we present a method to increase initial cell yield and to prolong culture of Schwann cell in proliferative phenotype. Sciatic nerves of adult Sprague Dawley rats were explanted and treated with extracorporeal shockwaves (ESWT). Subsequently Schwann cells were isolated and maintained for 8 weeks (passage every 6 days). Evaluation of proliferative phenotype was done with flow cytometry (P75, S100, P0) Schwann cells isolated from nerves treated with extracorporeal shockwaves showed an up to 50% higher initial cell number (normalized on sciatic nerve wet weight). Evaluation with flow cytometry revealed a significantly lower amount of myelin component P0 in the ESWT group compared to untreated control on day 19 after isolation. Cultured for up to 8 weeks (P6) Schwann cells treated with ESWT showed no change in marker expression (P75, S100) or proliferation, while marker expression of control group steadily decreased. Extracorporeal shockwave treatment of donor nerves shows beneficial effects on rat Schwann cell isolation and culture.

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Hyaluronan (HA) is a naturally occurring glycosaminoglycan and one of the chief components of the extracellular matrix, articular cartilage and synovial fluid, among other things. One of promising applications of HA is a hydrogel-based scaffold for articular cartilage regeneration used in the field of tissue engineering. In our previous works we designed a new HA tyramine derivative containing an alkyl-based linker for the preparation of noncytotoxic and biocompatible hydrogel-based materials with enhanced mechanical properties. In a following step we worked on optimization of the HA derivative parameters and the hydrogel properties to make the scaffold suitable for chondrocytes implementation and their subsequent cultivation. In this presentation we would like to present the results obtained from cultivation of porcine chondrocyte primoculture. The results show that chondrocytes implemented into the HA hydrogels are viable and that they produce an extracellular matrix, the quality and composition of which corresponds to articular cartilage tissue. Together with HA biological properties, these results indicate that our new designed HA hydrogels are optimal candidates for articular cartilage repair scaffolds.
Repair of bone defects is a difficult clinical problem for reconstructive surgeons. Bone tissue engineering using an appropriate scaffold with cells is a new therapy for the repair of bone defects. The aim of this study was to evaluate the \textit{in vitro} osteogenesis of canine adipose tissue-derived mesenchymal stem cells (Ad-MSCs) cultured in a combination of collagen I gel and a porous serum-derived albumin scaffold. A serum-derived albumin scaffold was prepared with canine serum by cross-linking and freeze-drying procedures. Ad-MSCs were seeded onto serum-derived albumin scaffolds with or without collagen I gel, and were exposed to osteogenic differentiation conditions \textit{in vitro}. After 28 days of \textit{in vitro} culture, distribution and osteogenic differentiation of Ad-MSCs cultured in the scaffold were evaluated using SEM, histology, immunohistochemistry, alkaline phosphatase (ALP) activity assay and calcium colorimetric assay. Ad-MSCs showed more homogeneous distribution and osteogenic differentiation in the scaffold with collagen I gel than without collagen I gel. ALP activity and extracellular matrix mineralization in the construct with type I collagen were significantly higher than in the construct without type I collagen ($P < 0.05$). The combination of collagen I gel and the serum-derived albumin scaffold enhanced osteogenic differentiation and the homogenous distribution of Ad-MSCs.
Limbal epithelial stem cells (LESCs) are responsible for corneal epithelium renewal. Their dysfunction cause corneal blindness and chronic pain. Current treatments include the in vitro cultured LESC transplantation. Unfortunately, LESC culture media (CM) often contain supplements, like cholera toxin, DMSO and FBS that can induce side effects. In addition, the number of limbal tissue donors is restricted, compromising both problems for this treatment. Here, three different CM were tested to limbal primary cultures (LPCs) cultivation: 1) CnT20 undefined-CM, 2) IOBA-FBS, a defined-CM with FBS in which cholera toxin and DMSO were replaced, and 3) IOBA-SH CM, IOBA-FBS CM with human serum instead of FBS. Besides, the same limbal tissue was consecutively cultured with IOBA-SH CM, obtaining LPC1 and LPC2. LPC characterization was performed. All LPCs cultured with the three CM presented cuboidal cell-morphology with no differences in the percentage of positive cells for limbal (ABCG2, p63 and K14) and corneal (K3 and K12) proteins. Conversely, except for K12, mRNA expression of these markers was higher in CnT20-cultured cells. The LESC marker K15 (protein and mRNA) was significantly more expressed with the IOBA-HS CM. The PECAM (endothelial cells), MART-1 (melanocytes) and CD11c (dendritic-cells) proteins were not expressed in any LPC, but fibroblast protein S100A4 was mildly detected with the three CM. The LPC1 obtained with IOBA-SH CM showed similar characteristics to the LPC0, while elongated cell morphology and a decrease in some LESC marker expression appeared in LPC2. Here we report an innovative CM (IOBA-SH) that enables the culturing of two bio-safe LPCs from the same limbal tissue that could potentially be used in clinical practice.
Mesenchymal Stem Cell Differentiation to Osteogenic Lineage by Bioactive Self-Assembled Peptide Hydrogels

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Conventional treatment strategies for bone disorders and wounds are not sufficient to heal bone tissue. Treatment methods including autografts, allografts or xenografts have several limitations such as patient pain, immune rejection risk and high cost. Regenerative medicine has great potential to eliminate the drawbacks of current therapeutic approaches due to its capacity to restore, maintain, or improve bone tissue function. One of the most promising strategies is to develop scaffolds mimicking functional components of extracellular matrix. Collagens and hydroxyapatite provide strength and flexibility. Glycosaminoglycans regulate growth factor activity and have significant roles in bone tissue formation. Enhanced bone tissue regeneration can be accomplished by mimicking extracellular matrix components such as collagenous proteins and glycosaminoglycans. For this purpose, a nanofibrous supramolecular system mimicking natural extracellular matrix structure and chemical composition of glycosaminoglycans was utilized for directing differentiation of mesenchymal stem cells into osteogenic lineage. Peptide amphiphile (PA) molecules were used for developing a nanofibrous supramolecular system. We observed that the peptide nanofiber system induced osteogenic activity of mesenchymal stem cells and calcium deposition. The peptide nanofiber hydrogel system offers novel platforms for selective induction of mesenchymal stem cells into osteogenic lineage, which might aid in the development of novel therapeutic approaches for treatment of bone defects.
Recent in vitro tissue engineering approaches have looked at incorporating chondrogenic and vascular priming and have found that separately, both processes have a positive effect on the osteogenic potential of MSCs [1, 2]. However, in vivo both are crucial for the development of bone tissue, and to date no in vitro tissue regeneration strategy has sought to incorporate both events. The objective of this study is to chondrogenically prime MSCs to form a cartilage template and subsequently pre-vascularise the constructs through coculture. To determine the optimum in vitro conditions for development of a cartilage template, pellets formed from MSCs harvested from BALB/c mice were chondrogenically primed for varying durations (10-28 days). Once the optimum conditions for the development of a cartilaginous template were determined, vascular priming was performed by co-culturing human bone marrow derived MSCs with HUVECs at a ratio of 50:50 per pellet. Biochemical, histological, and immunohistological analysis were performed. Our results show for the first time that chondrogenic priming for 14 - 21 days, prior to being exposed to osteogenic factors, produces the optimum conditions for the development of the cartilage template. Interestingly we can also see that the formation of the cartilage template provides a suitable platform for HUVECs to attach and invade. Moreover, our results show that the co-culture of the HUVECs and MSCs leads to an increase in osteogenic markers (ALP). This study shows that chondrogenic priming followed by vascular priming can enhance the osteogenic differentiation potential of MSCs.


Figure 2: (a) Alcian Blue stained sections; (b) Extracellular ALP activity expressed by co-culture pellets (*p<0.05 versus both groups at Day 20; n=3).
The integrity of the corneal epithelium is essential for clarity of vision. Normally, superficial epithelial cells are shed into the tear film and regenerated by epithelial stem/progenitor cells located at the limbus. Damage or depletion of the limbal cells leads to conditions such as limbal stem cell deficiency (LSCD). Current treatment for this condition involves transplantation of limbal epithelial cells expanded ex-vivo; however cell numbers are limited due to chronic ocular donor shortages. We have found a source of multipotent mesenchymal stem cells located in the corneal stroma that express CD34 and CD105 on isolation; expression subsequently diminishes during traditional tissue-culture plastic propagation. These cells show an ability to transdifferentiate into the corneal epithelial cell lineage. In this study, optimisation of culture conditions for maintained and efficient expansion of these cells is performed, using a range of media and culture environments. Early results suggest a three-dimensional environment and semi-solid medium intended for hematopoietic culture, demonstrate extended CD34 and CD105 expression. When cells were transferred to epithelial differentiation medium they showed epithelial morphology, significantly increased cytokeratin 3 and 19 expression, and considerable upregulation of genes related to corneal epithelial cells (ABCG2, DeltaN63, LEF1, HES1, FRZB1, KRT19, DTC and CDH1). This work will help produce methodologies to create cell banks from a corneal stromal stem cell source for generation of corneal epithelium, leading to improved surgical and visual outcomes in LSCD patients.
miR- 186 Targets the Pituitary Tumor Transforming Gene 1 (PTTG 1) and Regulates Migration of Human Oral Squamous Cell Carcinoma.

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Human pituitary tumor-transforming gene 1 (PTTG1), which is a newly identified proto-oncogene, over-express in many nonendocrine-related tumors as well as endocrine-related cancers. Although it has been known to involve in tumor metastasis, but the function and the regulation mechanism of PTTG1 on migration of oral squamous cell carcinoma (SCC) remain still unclear. Here, we analyzed the expression of PTTG1 in oral SCC and evaluated the mechanism for invasion of oral SCC cell lines (YD-10B and YD-15) depends on PTTG1 expression by siRNA. In addition, target microRNA (miR) which functions in transcriptional and post-translational regulation of PTTG1 expression was analyzed and evaluated. The invasiveness of oral SCC cells in Metrigel were significantly decreased after siRNA PTTG1 (siPTTG1) treatment comparing to Mock (p<0.05) without alteration of their cell cycle. Also, MMP-9 expression by siPTTG1 treatment was significantly decreased. Down-regulated PTTG1 in oral SCC cell lines induced to increase the phosphorylations of AKT and mTOR, otherwise, decrease the phosphorylation of STAT3. In addition, miR-186 expressed in all oral SCC cell lines and controlled the expression of PTTG1 when they were incubated with their mimic and inhibitor (P<0.05). Taken together, alteration of PTTG1 could be regulated migration of human oral SCC through the regulation of MMP-9 activity and miR-186 target their expression. Therefore, these results not only further our understanding of the potential roles of PTTGs in oral SCC cell lines, but also contribute to the foundation for a potential new treatment strategy using microRNA.

Keywords: oral squamous cell carcinoma, pituitary tumor-transforming gene, migration, siRNA, microRNA
Differential Response of Co-Cultured MSCs and Nucleus Pulposus Cells Encapsulated in Alginate and Chitosan Hydrogels under IVD-Like Nutrient Conditions

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Cell-based therapies have been proposed for the treatment of degenerated intervertebral disc (IVD). Given their propensity to proliferate and ability to form multiple tissue types, mesenchymal stem cells (MSCs) being proposed as a potential cell source to promote repair of the nucleus pulposus (NP). However, for any successful cell-based therapy a carrier biomaterial may be essential for targeted delivery and provide key biophysical and biochemical cues to facilitate differentiation of MSCs. Two widely used biomaterials for NP regeneration are chitosan and alginate. The key objective of this study was to assess the influence of alginate or chitosan hydrogels on bone marrow (BM) MSCs and NP cells in isolation or in co-culture.

Porcine NP and BM cells were encapsulated in alginate and chitosan hydrogels in separately at two seeding densities (\(4\times10^6\) and \(8\times10^6\) cells/ml) or in co-culture (1:1, \(8\times10^6\) cells/ml). Constructs (diameter:5mm, height:3mm) were maintained under IVD-like conditions (low-glucose, low (5%) oxygen) with or without TGF-β3 supplementation for 21 days. Results demonstrated differential viability depending on hydrogel type. NP cells remained viable in both biomaterial types whereas BM viability was diminished in chitosan. Furthermore, hydrogel type was found to regulate collagen deposition. Specifically chitosan suppressed and alginate promoted collagen accumulation. A key finding in this work was that alginate supported matrix accumulation in the absence of exogenous growth factor supplementation (TGF-β3) only when co-culture was employed. These findings provide exciting new insights on the potential of MSCs for NP tissue regeneration.
CD10, CD92 and CRYAB are Potential Novel Markers for Human Mesenchymal Stem Cells Undergoing Osteogenic Differentiation.

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Osteogenic differentiation of human mesenchymal stem cells (hMSCs) in vitro is commonly evaluated by the gene- or protein expression of multiple markers such as ALP, RUNX2 and OCN. However, there are no surface markers that define the population of hMSCs differentiating towards the osteogenic lineage. Such markers could be of great relevance in applications such as tissue engineering, for monitoring the maturation of a construct, or for cell therapy. Stable isotope labeling by amino acids in cell culture (SILAC)-based quantitative proteomics was utilized to investigate differently expressed surface markers in osteogenically differentiated and undifferentiated hMSCs. Membrane proteins, from labeled cells, were isolated and subsequently identified and their relative expression quantified by mass spectrometry (MS). Based on the MS results, the expression of a selected subset of proteins was validated by flow cytometry or ELISA. The MS analysis of the isolated proteins revealed 52 proteins with at least 2 times higher expression in osteogenically differentiated cells. Flow cytometry, established expression of the surface markers CD10 and CD92 as significantly increased in hMSCs differentiated towards the osteogenic lineage. Furthermore, validation by ELISA established the expression of the intracellular protein crystalline-αB (CRYAB) as significantly increased in osteogenically differentiated hMSCs. In conclusion, this study has identified differences in the proteome between osteogenically differentiated and undifferentiated hMSCs. The results demonstrate that the membrane proteins CD10 and CD92, and intracellular protein CRYAB are potential novel markers for the osteogenic differentiation of hMSCs in vitro.
Human mesenchymal stem cells (MSCs), which can generate both osteoblasts and chondrocytes, represent an ideal resource for tissue engineering approaches to orthopaedic repair. One major hurdle for the development of MSC-based osteochondral constructs is the difference in lineage-priming conditions: while serum is typically included in culture protocols promoting differentiation of the osteogenic component, typical chondrogenic differentiation protocols rely on the use of serum-free conditions. In order to define conditions compatible with osteochondral differentiation in a single vessel, we have analysed the efficiency of new in vitro approaches based on different serum exposure regimes across a range of treatment length and serum sources. Data generated from human MSC cultures indicate the suitability of dynamic treatment regimes, in which inclusion of a serum component is temporarily restricted to an early induction phase. Analysis of metabolic, cellular and phenotypic parameters confirmed the growth and differentiation potential of MSCs in such regimes, indicating possible new approaches to limit FCS exposure. Further in vitro evaluation revealed that although sera from different origins could be used to support MSC cells in culture, cells exhibited distinctive response patterns when analysing metabolic profile, marker activation, and differentiation response. Our study highlights the prospect of introducing new culture approaches based on tailored serum regimes, which may allow optimised cellular response for osteochondral tissue engineering using MSCs for regenerative medicine.
Prostate Carcinoma Cell Growth-inhibiting Hydrogel Supports Axonal Regeneration in vitro

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Prostate cancer is the third common malignant tumor in men. Radical prostatectomy, is the most commonly performed surgical therapy. Typically erectile dysfunction and incontinence, due to severing of the axons of the plexus prostaticus, are related side effects. To date, no reconstructive therapy is available. Here, we present an injectable hydrogel as a regenerative matrix that adapts to any given tissue topography. The two-component hydrogels (hydrolyzed collagen and transglutaminase) polymerized within 2 minutes and revealed an isotonic, microstructured network that could be tightly crosslinked to abdominal tissue in situ. Cell culturing demonstrated the biocompatibility of the gel and general cell permissiveness. No effect on cell adhesion, survival and proliferation of cells was observed. A chemotherapeutic drug integrated into the hydrogel reduced the risk of fibrosis and tumor relapse. Aversive fibroblast- and prostate carcinoma cell growth was inhibited, while axonal outgrowth from peripheral nervous system explants remained completely unaffected. In conclusion, these results suggest that the gel’s adequate viscoelastic properties and porous microstructure, combined with its tissue adhesion and neuritotrophic characteristics in the presence of a cell type-specific cytostatic, may constitute an appropriate hydrogel implant applicable to avoid prostatecto
Support of functional tissue regeneration requires a better understanding of cell growth behavior in scaffold pores. In this study, we aimed at getting an insight in the general pore-filling strategy of fibroblasts as the primary cell population building soft extracellular matrix (ECM) structures. Macroporous starPEG-heparin cryogels were seeded with human primary dermal fibroblasts and cultured up to 7 days to investigate pore closure. Staining for cell nuclei and actin cytoskeleton and fibronectin were performed to identify cell positions, cell organization and ECM formation over time. Cell number and remaining inner pore diameter were quantified and compared to a 2D geometrical model that describes cells as tensioned chords. To strengthen the validity of the model, cell growth inside well defined rectangular pores of a polymer mesh structure was analyzed by live cell microscopy for different dimensions. Good matching between cell numbers vs. open crosssectional area for all pore sizes was observed. Higher correlation coefficient between experimental data and model ($R^2=0.72$ to 0.94 for the different sizes) suggested preferential cell location at high substrate curvature. ECM first followed cell organization until reaching pore closure (Pearson's coefficient $p=0.45\pm0.01$) and subsequently got compacted in the center of the pore ($p=0.26\pm0.04$). The current chord model predicted the number of cells needed to fill individual pores and can be easily adapted to any initial scaffold geometry.
Development of a Human Extracellular Matrix for Regenerative Medicine Applications

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Interaction of stem cells with the extracellular matrix (ECM) is critical for their proliferation, differentiation and migration in culture. Our aim is to obtain a simple, low-cost method for generating a functional human ECM that obviates the use of feeders and animal products for stem cell culture. Confluent human foreskin fibroblasts (HFF) were lysed under conditions that retained their ECM as a coating on culture plates. After detailed characterization of the hffECM obtained, undifferentiated H9 and H1 cells were seeded and maintained over hffECM during 21 passages. To evaluate the capacity of ECM as support for hESC differentiation, adipose and endoderm precursor obtention were triggered. To further validate the functional properties of hffECM, cell migration was tested. H9 and H1 cells cultured on hffECM-coated plates expressed markers of pluripotency at higher levels than when cultured on human fibroblast feeders. Proteomics revealed that hffECM retains many of the components. hESC cultured on hffECM differentiated efficiently to adipocytes and endoderm precursors as well as cells that express muscle and neuron markers. The direction of migration was equivalent between hffECM and Matrigel, whereas the speed was enhanced on hffECM. Thus, ECM derived from human fibroblasts supports both pluripotency and differentiation of hESC, thereby revealing a novel and much-needed tool ECM supported cell based therapies.

Conclusions

We have developed a simple method that generates a biologically active human ECM under conditions that are compatible with clinical-grade studies. Our hffECM supports the maintenance and differentiation of hESC and based on our data, it can be applied to other types of stem cells that require a physical support.
Capillary Morphogenesis of Mono- and Co-cultures In StarPEG-Heparin Hydrogels for Controlled Vascularization

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Efficient vascularization of tissue-engineered constructs is essential for their successful integration and function upon implantation. We evaluated the angiogenic properties of recently developed in situ polymerizing starPEG-heparin hydrogel system crosslinked via Michael-type addition chemistry, incorporating integrin binding peptides, cell responsive crosslinkers and reversibly conjugated growth factors. Primary human umbilical vein endothelial cells (HUVECs) were embedded in hydrogels exploring a range of biophysical and biochemical properties. Using bright-field, confocal and electron microscopy, we observed that within 72 hours, endothelial cells vacuolized and coalesced into lumenized tubular structures, surrounded by basement membrane. Tube formation occurred primarily in soft scaffolds (200Pa) and was transiently induced for 5 days by vascular endothelial growth factor (VEGF165) at concentration dependent manner. In contrast to single factor delivery strategies, co-delivery of basic fibroblast growth factor (FGFb) and stromal derived factor 1-α (SDF1-α) synergistically increased the density and stabilized tubular networks up to 10 days. To further sustain the endothelial cell networks we established co-cultures with various mural cell types (bone marrow derived mesenchymal stem cells, smooth muscle cells or fibroblasts). An optimal ratio of 1:10 (mural:EC) resulted in a tubular network lasting for over 4 weeks. Our results indicate that starPEG-heparin hydrogels can effectively support vascularization and represent a promising tool for development of tissue engineered constructs.
Human Fibrochondrocytes Increase Biomechanical Properties of Novel Silk Fibroin Scaffolds for Meniscus Tissue Engineering

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Strategies for meniscus tissue engineering (TE) should be able to reply properties of native tissue. This work presents the properties and in vitro biological performance of novel silk fibroin scaffolds. Furthermore, the influence of seeding human meniscus fibrochondrocytes on biomechanical properties was assessed \textit{in vitro}. Silk-based scaffolds (10 and 12 wt\%) were produced by means of combining salt leaching and lyophilization methods. Human meniscus cells (HMC’s) were isolated using enzymatic digestion and expanded using standard culture conditions. Human fresh menisci macroscopically intact were used for cell’s harvesting and isolation. HMC’s were seeded at a cell density of $5 \times 10^4$ cells/scaffold. Cell-laden constructs were cultured in static conditions, up to 21 days. HMC’s adhesion was investigated by scanning electron microscopy (SEM). Viability (calcein-AM assay) and proliferation tests (DNA quantification) were performed until 21 days of culturing. Dynamic mechanical analyses (DMA) were also performed. SEM analysis revealed that HMC’s adhered to scaffold’s surface. Live/dead assay and DNA quantification analysis showed that HMC’s were viable and proliferated after culturing onto the 10 and 12 wt\% silk fibroin scaffolds. The moduli of acellular scaffolds immersed in culture medium for 14 days were $27.6 \pm 7.9$ kPa and $61.1 \pm 0.4$ at 10 Hz, for silk-10 and silk-12, respectively. The moduli of the cell-laden constructs after 14 days of culturing were $48.2 \pm 19.8$ and $140.1 \pm 15.6$ kPa, for silk-10 and silk-12, respectively. Silk scaffolds showed great promise for finding application in meniscus TE as it supported cells adhesion, proliferation and viability, and improved the biomechanical features of acellular scaffolds.
Obtaining a sufficient number of cells ex vivo for tissue regeneration, which are appropriate for cartilage repair, requires improved techniques for the continuous expansion of chondrocytes in a manner that does not change their innate characteristics. Rapid senescence or dedifferentiation during in vitro expansion results in loss of chondrocyte phenotype and the formation of fibrous cartilage replacement tissue, rather than hyaluronic cartilage, after transplantation. As demonstrated in the current study, wild-type p53-inducible phosphatase (Wip1), a well-established stress modulator, was highly expressed in early-passage chondrocytes, but declined rapidly during in vitro expansion. Stable Wip1-expressing chondrocytes generated by microporation were less susceptible to the onset of senescence and dedifferentiation, and were more resistant to oxidative stress. The increased resistance of Wip1 chondrocytes to oxidative stress was due to modulation of p38 mitogen-activated protein kinase (MAPK) activity. Importantly, chondrocytes expressing Wip1 maintained their innate chondrogenic properties for a longer period of time, resulting in improvements in cartilage regeneration after transplantation. Chondrocytes from Wip1 knockout (Wip1−/−) mice were defective in cartilage regeneration compared with those from wild-type mice. Thus, Wip1 expression represents a potentially useful mechanism by which a chondrocyte phenotype can be retained during in vitro expansion through modulation of cellular stress responses.
Tissue Engineering a Physiologically Relevant 3D in vitro Model of Colorectal Cancer

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Collagen type I hydrogels are commonly used for 3D cell culture as they provide a biomimetic environment in which to study cell behaviour. However, hydrogels do not accurately model tissue matrix density in vivo. Increasing collagen density alters scaffold properties such as matrix stiffness, a crucial parameter governing cell behaviour and can help recreate normal tissue barrier function in vitro. With this in mind, we developed a spatially accurate 3D in vitro model of colorectal cancer known as a ‘tumouroid’ that recapitulates the dense architecture of tumours in vivo. We engineered 3D tumouroids of varying collagen density by using plastic compression¹. These tumouroids contained colorectal cancer cells (HT29 or HCT116), and were placed into a surrounding collagen hydrogel, which mimicked the stromal component of the tumour environment. Characterization of this model included investigations into cell growth (Alamar Blue®) and invasion, as well the expression of EGFR (Epidermal growth factor receptor) by immunofluorescence. Matrix density was 2.63% ± 0.16% and 8.84% ± 1.24% for partially compressed or fully compressed collagen type I hydrogels respectively (%v/w). Cells survived for 14 days in culture and formed 3D cellular spheroid aggregates. Immunofluorescence confirmed expression of EGFR by multicellular aggregates. Our 3D in vitro cancer model exhibits more physiologically relevant matrix densities. This is essential for a model that will enable us to study the diffusion gradients that exist within tumours and how they affect both cell behaviour and the responsiveness of cells to cytotoxic treatment.

![Image of EGFR expression in 3D cellular aggregates](image)

**Figure 1:** EGFR expression (red) in 3D cellular aggregates of (a) HCT116 (b) and HT29 cells after 14 days in fully compressed collagen type I gels. (Blue stain – DAPI - cell nuclei)

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Effect of Biomimetic Modification of Porous Polypeptide-Based Hydrogels on Stem Cell Chondrogenesis

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Soft porous hydrogels based on biodegradable poly(α-amino acids) (PAA) were prepared as a candidates for cartilage regeneration. The main benefit of synthetic PAA is that peptide bonds could be cleaved by enzymes present during remodeling processes in tissues. In this work we studied the rate of RGDS incorporation to the hydrogel and the effect of biomimetic modification on human tooth germ stem cells (HTGSCs) adhesion, proliferation and chondrogenic differentiation. The covalently crosslinked gels were formed by radical copolymerization of methacryloylated macromonomer poly(N5-(2-hydroxyethyl)-L-glutamine-stat-L-alanine-stat-methacryloyllysine] as a multifunctional macromonomer with 2-hydroxyethylmethacrylate as minor comonomer using 2,2´-azobisisobutyronitrile as initiator. Generated pores in the range 5–20 micrometers were formed by phase separation of small fraction of polyhydroxyethylmethacrylate. The efficiency of incorporation of RGDS sequence was studied using iodine radiolabeled peptide via Chloramine-T radioiodination reaction as one step in solid peptide synthesis. The effect of biomimetic modification of hydrogels with adhesive peptide RGDS on the seeding efficiency of HTGSCs was studied in vitro. While unmodified hydrogels showed very low cell adhesion due to their highly hydrophilic nature, the incorporation of adhesive peptides significantly improved adhesion and viability of seeded cells. The Alcian Blue staining confirmed that the HTGSCs in the biomimetically modified scaffolds produced more sGAG, which means that the cells could undergo chondrogenic differentiation. Acknowledgement to financial support of P108/12/1538 and ASCR-TUBITAK(111M031).
Bimolecular-Based Self-Assembling Hydrogel for Application in Cartilage Repair

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Adult articular cartilage has a very limited capacity of regeneration. Therefore, treatments to assist cartilage repair is a challenge topic in regenerative medicine. This work aims to mimic cartilage extracellular matrix using a new bi-component scaffold produced by a simple combination of oligosaccharide molecules of heparin and the commercial available self-assembling peptide PuraMatrix. This bi-molecular hydrogel was used to foster chondrogenic differentiation in vitro culturing Mesenchymal Stem Cells (MSCs). Particularly, human Adipose Derived Stem Cells (ADSC), a population of adult stem cells able to differentiate into mesenchymal derived tissues such as bone, cartilage and fat, were used to study the potential properties of the new material. First, we obtained an optimal working mix range of heparin and PuraMatrix to ensure the self-assembling process and the most homogenous mixture of the two components. Then, ADSC were cultured using different heparin/Puramatrix combinations and good cell viability was observed after four weeks of culture. Interestingly, during the first days of culture the three-dimensional (3D) constructs underwent a pronounced diameter reduction that ended in a small compact structure with an apparent increased stiffness. This morphological change was prompted partly due to the chondrogenic induction media which contains: recombinant human transforming growth factor-β1 (TGF-β1), dexamethasone and L-Ascorbic Acid 2-phosphate (AA2P). In addition, the induced constructs become highly stained with toluidine blue dye, indicating the presence of synthetized proteoglycans. Moreover, specific markers of mature cartilage such as collagen type II and aggrecan were detected by Reverse Transcription Polymerase Chain Reaction (RT-PCR) and Western Blot analyses. These results suggest that this simple approach to obtain three-dimensional (3D) scaffolds seems to recreate the required microenvironment for the cells to differentiate to chondrogenic lineage.
Preliminary Study to Determine the Effect of Narrow Band Red Light on Cell Number and Metabolic Activity

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Introduction. Age-related Macular Degeneration (AMD) is a blinding eye disease due to loss of Retinal Pigmented Epithelium (RPE) function which supports the neural retina. The aim was to determine the effect of red light (RL) on RPE cell proliferation and metabolism as a potential future therapy.

Methods. Sub confluent cultures of ARPE-19 cells were exposed to narrow band 633nm emission RL at 0.1 or 0.2 W/sr/m² for 2, 4, 8, or 24 h. Control cultures were exposed to UV or no light (NL) stimuli. A resazurin conversion assay was used to measure total cell metabolism at 0 (T0), 24(T24), 48(T48) and 144(T144) h post exposure and quantified in a fluorescent plate reader. Samples were then fixed and stained with DAPI and phalloidin. ImageJ was used to quantify cell number and analyse morphology.

Results. No significant difference was observed in cell number between samples post 2 h RL exposure at any time point. UV exposure for 4 and 8 h reduced the cell number but not to a statistically significant level. 24 h UV exposure caused a decrease in cell number by T24 which was significantly reduced by T48 (see fig.). An increased metabolic activity was measured at T24 and T48 post 2, 4 and 8 h RL & UV exposure but was not significantly different from NL exposure.

Conclusion. RL exposure caused a slight increase in metabolic activity in the cells although this was typically not significantly different from NL conditions. Only UV exposure for 24 hours caused a significant reduction in cell number.
In Chronic Lymphocytic Leukemia Nurse Like-Cells Are Targeted By Hepatocyte Growth Factor Produced by Bone Mesenchymal Stromal Cells

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Introduction
The stromal cell secretoma is of paramount relevance for the bone marrow signal network. Hepatocyte growth factor (HGF) secreted by stromal cells and present in sera of chronic lymphocytic leukemia (CLL) patients supports the persistence of leukemic B cells, interacting with cMET, its transmembrane kinase receptor. However, targeting other cell types in the marrow niche may influence CLL relapse. Since also the nurturing population of nurse-like cells (NLC), bystanders in CLL lymphoid tissues, sustains the leukemic B clone, we evaluated if NLC could represent HGF additional targets.

Materials and Methods
Freshly-prepared or cultured PBL mononuclear cells from healthy or CLL donors were grown for 2 weeks (6x10^6 cells/ml of complete medium); after B cells removal, adherent NLC were detached for flow cytometry/mRNA extraction; their cMET expression was assessed by real time RT-PCR and immunocytofluorescence; hrHGF was used to stimulate cultured NLC for 48 hrs to evaluate phosphorylation of STAT3, a downstream effector of cMET.

Results
A high cMET mRNA level was detected in NLC, doubling that of B cells. Moreover, in cytofluorimetric assays, the cMET-dependent mean fluorescence intensity in monocytes proved stronger in CLL patients than in healthy subjects. Cytoimmunostaining and flow cytometry revealed STAT3^TYR705 phosphorylation upon HGF/cMET interaction, confirming an active role of the HGF/cMET axis in NLC.

Conclusions
In NLC we proved a sustained expression of cMET and its role in STAT3 phosphorylation. This prompts NLC as functional targets for the HGF of the stromal secretoma. These results add new insights to bone marrow cross-talk and to tissue engineering/therapy approaches of mesenchymal/lymphoid tissues.
Tissue-engineered implants are commonly applied for the treatment of localized cartilage lesions. The goal of this work is to determine and establish biomolecular parameters accounting for the quality of collagen I-matrices seeded with autologous chondrocytes from a local biotech company. A special focus is set on cartilage-constituting, matrix-degrading, and cell surface proteins since they might influence the clinical outcome after implantation. To characterize the chondrocytes in the transplants we detect chondrocyte-specific markers (e.g., Aggrecan, Collagen 1A1, 2A1, 2AB, MMP 3, 13, Sox5, Sox6 and Sox9 by means of quantitative Real-Time-PCR (qPCR).

To confirm the results of the (qPCR) we concentrate on surface markers to detect the chondrogenic capacity of the chondrocytes in the transplant by means of flow cytometry (FACS). There are well known surface markers like CD44 and CD151 which are expressed significantly higher in chondrocytes with a higher chondrogenic capacity. Whereas chondrocytes with low chondrogenic capacities express on the cell surface high amounts of CD90, CD105 and CD166.

This will enable to establish a panel of cellular markers that help to evaluate and to determine the quality of tissue-engineered matrices. In addition, the knowledge of the critical parameters determining a positive clinical outcome will facilitate the optimization of cell-seeded matrices.
Potential Role of Matrix Metalloprotease Activity Involved in Cellular Migration in the Mammal Intervertebral Disc Region

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Introduction: Disc degeneration is believed to play a causal role in chronic lumbar pain. Regeneration processes and cellular migration in the intervertebral disc (IVD) have been sparsely described. Recently, potential stem cell niches in IVD regions and a cellular migration route (MR) were identified (rabbit model). Our aim was to gain further knowledge of IVD regeneration by examining migration mechanisms, potential matrix metalloprotease (MMPs) involvement and matrix remodeling during cellular migration from niche regions toward the IVD (normal). Further, to examine the presence of MMPs in human (degenerated IVDs).

Material and methods: In total, IVD tissues (degenerated) were collected: 6 human donors (age 34-50 years), 2 mini pigs (normal, age 6 months) and 7 rabbits (normal, age 3 and 13 months). Human, (n=3), porcine (n=2), lapine (n=4) were examined by IHC for: MMP13, MMP9, IL1β (matrix degradation), SNAI1, SLUG (migration) and β1-INTEGRIN (cellular adhesion), (GDF5, prechondrocytic marker). Further, markers were analyzed by FACS (lapine, n=3). Additional samples were analyzed by Real-time PCR for the same markers (lapine, n=3, human, n=3).

Results: MMP9, MMP13, IL1β, SLUG, SNAI1, GDF5 (lapine, porcine, human) and B1-INTEGRIN (lapine, human) were found (protein level) in PMR and IVD regions. Gene expression of the same markers was detected in IVD tissues (lapine, human).

Conclusions: Results suggest involvement of MMPs, matrix remodeling and the presence of immature GDF5* cells in MR during migration of cells (originating from stem cell niches) towards the IVD. This may be important for understanding regenerative/growth mechanisms in the IVD in a perspective of future development of biological treatment strategies.
Development of Fully Defined Xeno-Free Culture System for the Preparation and Propagation of Cell Therapy Compliant Human Adipose Stem Cells

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Introduction: Adipose tissue is an attractive and abundant source of multipotent stem cells. Human adipose stem cells (ASCs) have shown to have therapeutic relevancy in diverse clinical applications. Nevertheless, expansion of ASCs is often necessary prior to clinical use, yet standard in vitro cell culture techniques utilize animal-derived reagents that should be avoided due to safety concerns. Therefore, xeno- and serum-free (XF/SF) reagents are highly desirable for enhancing the safety and quality of the transplanted ASCs.

Materials and methods: In the current study, animal component-free isolation and cell expansion protocols were developed for ASCs. StemPro MSC SFM XF medium with either CELLstart CTS coating or Coating Matrix Kit were tested for their ability to support XF/SF growth. Basic stem cell characteristics such as immunophenotype, proliferation and differentiation potential were assessed in XF/SF conditions and compared with human serum (HS) or traditionally used fetal bovine serum (FBS) cultures.

Results: ASCs cultured in XF/SF conditions had significantly higher proliferation rate compared to HS/FBS cultures. Characteristic immunophenotype of ASCs was maintained in every condition; however cells expanded in XF/SF conditions showed significantly lower expression of CD54 (ICAM-1) at low passage number. Further, multilineage differentiation potential of ASCs was maintained in every culture condition.

Conclusions: Our findings demonstrated that the novel XF/SF conditions maintained the basic stem cell features of ASCs and the animal-free workflow followed in this study has great potential in clinical cell therapies.
In vivo Grafting of Muscle Stem Cells Cultured on Gelatin-Genipin Hydrogels

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Introduction: Collagen VI is a large protein located in the basement membrane of skeletal muscle, where it plays key roles in the homeostasis of myofibers and in the self-renewal of adult muscle stem cells, or satellite cells (SCs). When collagen VI is genetically ablated in \textit{Col6a1}\textsuperscript{−/−} (KO) mice, muscle regeneration is impaired and the ability to preserve SC pool compromised [2]. Matrix stiffness may be a key mechanism through which collagen VI exerts its effects on SCs.

Experimental: Mechanical properties of wild-type (WT) and KO tibialis anterior (TA) muscles were measured \textit{in vivo} and mimicked \textit{in vitro} by hydrogels composed of gelatin cross-linked with genipin. To evaluate the effect of stiffness on SC stemness, WT SCs were cultured on such substrates, then SCs were detached and injected into KO TA to assess SC fate.

Results: KO TA muscles show reduced elastic modulus (7 kPa) compared to WT TA (12 kPa). SCs cultured on physiological stiffness were more able to preserve the Pax7 marker compared to SCs grown on pathological stiffness. When detached and injected in KO TA, the presence of donor SCs led to induction of muscle regeneration, but the total number of SCs was significantly higher in muscles transplanted with SCs grown on 12 kPa compared to SCs grown on 7 kPa. Interestingly, SCs grown on physiological stiffness were able to adopt a stem localization under basal lamina.

Conclusion: These data support the concept that collagen VI regulates the stemness and self-renewal of SCs through the fine regulation of muscle stiffness. Hydrogels with defined stiffness represent a useful method to reproduce SCs behavior both \textit{in vitro} and \textit{in vivo}. 
Changes in Human Skin-Derived MSC Phenotype and Mitochondrial Membrane Potential During Neuroectodermal Differentiation In Vitro


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Introduction
Skin derived mesenchymal stem cells (S-MSC) are multipotent cells representing promising material for regenerative medicine. We used two different protocols to differentiate S-MSC towards neuroectodermal lineages and assessed changes in neuroectodermal gene expression, growth factor secretion and mitochondrial membrane potential (MMP).

Materials and methods Following media compositions were used: 1) control – DMEM with 10% FBS; 2) preinduction – DMEM/F12 3:1, 2% B27, FGF and EGF (20 ng/ml ); 3) neuronal differentiation (ND) – Neurobasal, 2% B27, 1% N2, NT-3, BDNF (50 ng/ml) and 4) glial differentiation (GD) – DMEM/F12 3:1, 2% B27, 50 ng/ml neuregulin, 4µM forskolin. Media was changed every 3 days over three week experiment. We used JC-1 to evaluate changes in cell MMP on FACScalibur and calculated results as FL2:FL1 median fluorescence ratio. PCR methods were used for gene expression and ELISA to assess changes in FGF, VEGF and SDF-1 secretion.

Results
Immunofluorescence and PCR data suggest that several markers, notably, Nestin, Tubulin bIII, GFAP and SOX10 are expressed in differentiated and naïve cells. Secretion of SDF-1 increased significantly from 5104±297 pg/ml in control to 10137±420 in ND and 14055±4112 pg/ml in GD medium. VEGF secretion increased in GD media from 1954±177 to 3500±158 pg/ml. MMP (FL1:FL2 ratio) increased more than 2-fold in pre-induction media (from 0.66±0.35 to 2.3±0.04) and was consistently higher in GD media compared to ND media during differentiation process (0.96±0.4 vs 0.21±0.28).

Conclusions
S-MSCs express neuroectodermal markers. Secretion of SDF-1 and VEGF that are involved in angiogenesis and neuroprotection is increased during differentiation. We have detected considerable changes in MMP, thus, assessment of mitochondrial function could be used to determine state of differentiation.
Adipose stem cells (ASCs) are a promising autologous cell source for the applications of regenerative medicine. They are multipotent stem cells able to differentiate in vitro for example towards bone, cartilage and fat cells. In bone tissue engineering the viability and differentiation of adipose stem cells has been widely studied with different biomaterial compositions. However, the exact molecular mechanisms behind biomaterial-induced molecular and functional changes in ASCs are largely unknown. The aim of this study was to enlighten cell attachment related intracellular signaling mechanisms when ASCs were cultured on bioactive glass (BaG, S53P4) discs and granules. Specifically, we persisted to analyze the integrin-focal adhesion kinase (FAK)-mitogen-activated protein kinase (MAPK) signaling cascade in biomaterial interacting ASCs. Our results indicated that ASCs remained viable on the biomaterials studied. Additionally, BaG biomaterials elevated levels of integrin expression and phosphorylation of FAK and MAPK p38. In the ERK phosphorylation, no clear differences were observed between the biomaterials and the control. However, when we further analyzed the role of ERK activation for the osteogenic potential of BaG cultured ASCs, we noticed that ERK inhibition clearly hindered the BaG-induced osteogenesis. These results strongly imply that BaG has osteogenic potential and thus support its use in bone tissue engineering.
Optimal Autologous Culture of Endothelial Progenitor Cells for Tissue Engineering of Vascularized Implants

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The sufficient supply with nutrients and growth factors is of critical value for tissue engineered implants of large size. This can be achieved by promotion of neovascularization within scaffolds. Human bone marrow derived endothelial progenitor cells (EPC) can serve as an autologous source of cells promoting neovascularization. Here, we aim to optimize the isolation and culturing procedure of EPC with regard to their proliferation capacity as well as their endothelial differentiation capability. EPC were isolated from human bone marrow using Ficoll. EPC (CD34+/CD133+) were enriched using MACS\textsuperscript{®} Technology. Cells were cultured in IMDM supplemented with varying concentration of autologous platelet-derived growth factors and FCS. Commercial endothelial growth medium and IMDM supplemented with recombinant VEGF were used as control. EPCs were characterized from passage 1 to 4. The expression of endothelial markers was determined by real-time PCR and flow cytometry. In addition, we used FACS to evaluate lectin binding and uptake of Dil-labeled acLDL by EPC. Tube-formation capability was analyzed on MATRIGEL\textsuperscript{™}. Here we demonstrate that IMDM supplemented with autologous platelet-derived growth factors was highly efficient to promote vessel formation, whereas recombinant VEGF alone failed to trigger tube formation in MATRIGEL\textsuperscript{™}. In addition, a minimal content of FCS was proven better. These results demonstrate that EPC may trigger neovascularization in tissue engineered implants. Most importantly, we present an autologous culture method of EPC enabling a potential translation into clinics.
Previously, we have demonstrated that when MEFs were cultured in a non-instructive soft nanofiber scaffold (RAD16-I) under certain biomechanical and biophysical conditions, they underwent spontaneous chondrogenic differentiation characterized by the expression of chondrogenic markers (Quintana et al. 2009). Hence, in order to understand the possible molecular mechanisms regulating the default cartilaginous commitment, we studied the expression of genes involved in early tissue organization such as Noggin and Bmp4. Surprisingly, it was observed an up-regulation of both genes at 5 days of culture only when cells were cultured at low stiffness values. We speculate that the system autonomously regulates cartilage tissue formation by a mechanism involving early Bmp4 inhibition by Noggin.

Moreover, we were curious to see if the system undergoing spontaneous chondrogenesis was sensitive to change to an osteogenic fate by exposing it to a natural biological signal. Thus, we challenge it to respond to the presence of endothelial cells in a co-culture system. Interestingly, it was observed an up-regulation of the hypertrophic marker Collagen X and a localized mineralization of the ECM at the interface between both cell types. These results suggest us that the cross-talk between MEFs and HUVECs in vitro could be recreating some aspects of the endochondral ossification process.
Integration of orthopedic and dental implants into the existing tissue (osseointegration) is a major problem. In order to facilitate proper bone healing in the long term, migration of mesenchymal stem cells to the implant-tissue interface and their differentiation into bone producing osteoblasts are crucial. In order to accelerate this process, a common strategy is to modify surface properties to guide these cells into osteogenic differentiation. Herein, we demonstrate biofunctionalization of titanium surfaces through a mussel-inspired adhesion mechanism. Supramolecular peptide nanofibers presenting bioactive peptide sequences can induce hMSCs differentiation into osteogenic lineage. These nanofibers enabled deposition of bone-like hydroxyapatite (HAP) in the presence of simulated body fluid, a reminiscent property of type I collagen, and the major organic component of bone tissue. We characterized the HAP deposited nanofibers using Raman spectroscopy, XRD, electron diffraction, SEM, and EDS. We then evaluated the osteogenic differentiation of hMSCs on these organic-inorganic hybrid interfaces through both biochemical and molecular analyses. Our results showed that hMSCs differentiated into mature osteoblasts by use of ECM mimetic bioactive peptide networks.
Mesenchymal stem cells (MSCs) are mesoderm-derived cells that are considered a good source of somatic cells for treatment of many degenerative diseases. Previous studies have reported transdifferentiation of mesodermal MSCs into several endodermal and ectodermal cell types beyond their embryonic lineages, including hepatocytes, pancreatic beta cells, and neurons. However, the molecular pathways responsible for the direct or indirect cell type conversion and the functional ability of the differentiated cells remain unclear and need further research. In the present study, we demonstrated that valproic acid (VPA), which is a histone deacetylase inhibitor, induced an increase in expression of endodermal genes including CXCR4, FOXA2, SOX17, and GSC in human umbilical cord-derived MSCs (hUCMSCs). Moreover, we found VPA is able to increase these endodermal genes by activating signal transduction of ERK1/2 and AKT1. The resulting VPA-treated endoderm-like hUCMSCs were effectively differentiated into cells displaying multiple features of hepatic cells. Additionally, the effects of VPA on modulating hUCMSC fate were diminished by blocking AKT1 activation using specific signaling inhibitors of ERK1/2 and AKT1, PD0325901, and LY294002. Together, our results suggest that VPA contributes to the lineage conversion of hUCMSCs to hepatic cell fate by modulating expression of endodermal genes through AKT1 activation. [This research was supported by the Bio & Medical Technology Development Program of the National Research Foundation (NRF) funded by the Korean government (MEST) (2012M3A9B4028636)]
Engraftment Potential of Spheroid-forming Hepatic Endoderm Derived from Human Embryonic Stem Cells

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Transplantation and drug discovery programs for liver diseases are hampered by the shortage of donor tissue. While recent studies have shown that hepatic cells can be derived from human embryonic stem cells (hESCs), few cases have shown selective enrichment of hESC-derived hepatocytes and their integration into host liver tissues. Here we demonstrate that dissociation and reaggregation procedure after an endodermal differentiation of hESC produces spheroids mainly consisted of cells showing hepatic phenotypes in vitro and in vivo. A combined treatment with Wnt3a and BMP4 efficiently differentiated hESCs into definitive endoderm in an adherent culture. Dissociation followed by reaggregation of these cells in a nonadherent condition lead to the isolation of spheroid-forming cells that preferentially expressed early hepatic markers from the adherent cell population. Further differentiation of these spheroid cells in the presence of hepatocyte growth factor, oncostatin M, and dexamethasone produced a highly enriched population of cells exhibiting characteristics of early hepatocytes, including glycogen storage, indocyanine green uptake and synthesis of urea and albumin. Furthermore, we show that grafted spheroid cells express hepatic features and attenuate serum aspartate aminotransferase level in a model of acute liver injury. These data suggest that hepatic progenitor cells can be enriched by the spheroid formation of differentiating hESCs and that these cells have engraftment potential to replace damaged liver tissues. [This research was supported by the Bio & Medical Technology Development Program of the National Research Foundation (NRF) funded by the Korean government (MEST) (2012M3A9C7050139)]
Embryonic stem cells (ESCs) possess the capacity for self-renewal and pluripotency, and are able to differentiate into many different cell types. However, regulatory mechanisms underlying the differentiation of ESCs into specific cell types are poorly defined, and thus understanding its mechanism might allow us to manipulate the stem cell fate for stem cell-based therapies. Hypoxic microenvironment plays an important role in the proper embryonic development. The effects of low oxygen levels on stem cell fates (self-renewal and differentiation) are still not clear and controversial. We investigated whether hypoxia (1% O_2) efficiently directs the cultured mouse ESCs to differentiate into the specific lineage, especially the mesoderm-lineage, and, if it does, the underlying molecular mechanisms. We used differentiation cultures that make mESC develop into embryoid bodies (EBs) to mimic early embryonic development. Here, we have shown that a short time hypoxic exposure of EBs enhanced differentiation toward meso-endodermal cells, which differentiated into vascular-lineage and muscle-lineage cells more efficiently than normoxic EBs did. First, hypoxic treatment decreased the pluripotency through Oct4 suppression. Second, hypoxic exposure of EBs highly increased VEGF, an endothelial growth and differentiation factor, thereby successfully differentiated them to vascular-lineage. Also, hypoxic EBs could efficiently differentiate to muscle-lineage cells such as connexin43-positive cells. Moreover, transplantation of hypoxia treated EBs into mouse ischemic limb showed enhanced vessel- and muscle-differentiation. Our findings proposed hypoxic exposure of ESCs might be an efficient means of inducing ESCs into specialized lineages such as mesoderm-commitment.
One-Stage Tissue Engineering of Bladder Wall Patches for an Easy-To-Use Approach at The Surgical Table

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INTRODUCTION: Culturing autologous urothelial cells and transplanting them can be considered an appropriate method to restore bladder tissue. Drawbacks include the demand of in-house cell culture facilities and laborious procedures which limits its use in ordinary surgical units. In vivo expansion of bladder mucosa can be considered an alternative. The aim of this study was to construct transplants with high tensile strength with autologous proliferating cells that could be used for reconstruction of bladder tissue.

METHODS: Plastic compression of two cubes of collagen type I gel including a polycaprolactone (PCL) knitted fabric was carried out by a technique previously described. Minced bladder mucosa was seeded on top of the scaffold. The final construct was cultured in vitro for evaluation of cell expansion and re-organization in ordinary cell culture conditions. Constructs were analysed up to 6 weeks after initiation in respect to morphology, histology and imaging with scanning electron microscopy.

RESULTS: We showed successful proliferation of urothelial cells upon the scaffold in vitro. After only two weeks a single cell layer was formed and after 4 weeks a multi-layered epithelium. The cells were viable and proliferative with morphological features characteristic of urothelial cells. The construct had high tensile strength (17.9±2.6 Mpa).

DISCUSSION & CONCLUSIONS: By adding minced urothelial tissue to a collagen gel including a PCL knitted fabric, cell expansion and re-organization of epithelium could take place without the need for conventional in vitro cell culturing. The method is simple and could be used as a one-staged procedure in an ordinary surgical unit and could be a future approach for bladder augmentation.
Collagen-Based Scaffolds for Three-Dimensional Airway Models

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Improved, physiologically-relevant three-dimensional (3D) airway models are required to advance respiratory drug development and to provide effective replacement implants in cases of extensive tracheobronchial damage. Tissue-engineering strategies offer a means to support long-term growth and differentiation of respiratory epithelia, including co-culture, that facilitates improved airway modelling and tissue regeneration. In this study, we fabricated a porous 3D collagen-glycosaminoglycan (CG) scaffold and investigated its potential to support the growth and differentiation of a functional airway epithelium. Freeze-dried scaffolds were seeded with Calu-3 bronchial epithelial cells for culture at either an airway-liquid interface (ALI) or liquid-liquid interface (LLI). DNA quantification revealed that Calu-3 cells proliferated on the scaffolds with significantly increased growth at ALI culture. Immunostaining and histological analysis detected the presence of tight junction protein and mucin, all indicative of barrier formation. We predict that on-going PCR and electrophysiological studies will corroborate this evidence. Overall, we have shown that the CG scaffolds can support the proliferation and differentiation of airway epithelial cells, particularly in ALI culture, and provide effective templates for further scaffold design and tailoring as \textit{in vitro} airway models. These models also have the potential to be developed as a medical device for proximal airway tissue regeneration.
Mesenchymal stromal cells (MSCs) have been shown to improve tissue regeneration in several pre- and clinical trials. These cells have been used in combination with three-dimensional scaffolds as a promising approach in the field of regenerative medicine. In this work, we compare the behavior of adipose derived MSCs seeded on four different biomaterials that are commonly used or emerging in clinical settings. MSCs derived from human lipoaspirates were isolated, characterized, and seeded onto scaffolds constructed from bovine collagen, fibrin, chitosan, and decellularized porcine dermis. Results showed that the composition of the scaffolds strongly influences key parameters of the cells such as, seeding efficiency, cellular distribution, attachment, survival, metabolic activity, and paracrine release. This work provides information for clinical translation and represents the first step for optimizing the use of MSCs in FDA approved biomaterials in tissue regeneration. However, further studies need to be performed to evaluate their significance in in vivo models.
Using of Poly-Thiophene and Collagen for cell signaling in Liver Tissue Engineering

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Liver tissue engineering (LTE) requires a suitable extracellular matrix (ECM) for hepatocyte cell culture due to strict requirement of anchorage-dependent growth. Chitosan (CS), a partially deacetylated derivative of chitin polymer is highly used due to its high biocompatibility, non-toxicity, biodegradability and non-immunoreactivity to the human body compared to most synthetic polymers. On the other hand, Gelatin is partially hydrolyzed form of Collagen and a crucial component of the ECM. It has great ability to enhance cell attachment [4]. Poly-Thiophene (PTh) has recently been explored as an alternative to PPy, as it is much more stable to oxidation and is more conductive. It has been shown to retain 89% of its conductivity under similar conditions compared to PPy. Also type I Collagen, the most important constituent of ECM in mammals, is a suitable scaffold material for generating artificial substitutes for diseased or damaged tissue, especially for soft tissues and organs. In the present study, we use the composite of these materials as a new scaffold for liver tissue engineering. Gelatin at 6.4% (w/v) concentration was poured in a 5% Acetic Acid solution. Then, Chitosan mixed with a 2.1% (w/v) concentration via homogenizer at 29,000 rpm, using a cold water bath (Sample A). Collagen (I) and PTh were added with 0.0, 0.1, 0.2 and 0.3 (w/v) (respectively samples A, B, C and D). Afterward, the solutions were frozen and after been treated by freeze-lyophilization at -53°C and 0.05 milbar for 24 hours, the scaffolds were immersed in 50 mM 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC)/ 8mM N-hydroxysuccinimide (NHS)/ 95% alcohol crosslinking solution at 4°C twice for 48h. The cross-linked scaffolds were freeze-dried again under the same condition. Further studies on equilibrium swelling, Porosity measurement, In vitro degradation, FTIR spectra and X-ray diffraction, Thermogravimetric (TG) and Differential Scanning Calorimeter (DSC) analysis and scaffold morphology (SEM) were done. Biocompatibility of the scaffolds was evaluated by Cell attachment and Proliferation (by using GS5 hepatocyte cells), and protein detection (Western blot). The freeze-dried samples showed a range of 150-280 micrometers in porosity width and more than 1000% water swellability after six hours. The FTIR spectra illustrate the functional groups of each part with extra bonds between them. Finally the highest amounts of cells in attachment and proliferation tests were observed on the surface of the sample “C”. Also the cells in sample “C” expressed much more Alphafetoprotein and Actin.
MSC-Osteoblast Crosstalk in Osteogenesis

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In bone fracture repair, osteoblasts and MSCs attracted by cytokines are the two cell types mainly responsible for new matrix formation and calcification. MSC-osteoblast signalling has therefore been under investigation in vitro through co-culture studies. Although these two different cell types are both implicated in the osteoblast lineage, the optimum culture conditions and the cross talk between the cells remain unclear. We found that 1% FBS containing osteogenic medium allows higher levels of calcification in human osteoblasts compared to higher serum concentrations. Not only was the amount of calcification serum-dependent but it could also be correlated with the number of population doublings per day during the expansion period. The same holds true for ALP upregulation, albeit the peak could be seen in higher serum concentration. TGFβ-1 reduced calcification and this effect was not serum dependent. Fast growing osteoblasts have a high calcification potential and could induce hMSCs to calcify in a well-insert co-culture system in monolayer. The strength of induction correlated to the calcification potential of the osteoblasts alone. The paracrine effect is reduced with increasing serum and mostly localized to the area directly under the permeable insert. This strongly supports the idea of a soluble paracrine factor being secreted by osteoblasts to induce MSC calcification under low serum conditions.
**Introduction**

Mesenchymal stromal cells (MSC) are supposed to be beneficial for therapeutic revascularization of ischemic tissues and for support of vessel formation in engineered tissue constructs. Here we investigated, if MSC derived from blood vessels (bv-MSC) exert similar or different effects on endothelial cells (EC) as MSC derived from avascular tissues (av-MSC).

**Materials and Methods**

av-MSC were isolated from the placental amnion, bv-MSC and EC from placental blood vessels. The effects of conditioned medium (Cdm) on EC viability and network formation were determined using LDH and Matrigel assay, respectively. Angiogenic factors in Cdm were analysed by angiogenesis arrays and ELISA.

**Results**

Cdm from av- and bv-MSC induced a higher viability of EC (41%) than EC-Cdm (29%) compared to control medium. In the Matrigel assay, av-MSC-Cdm and EC-Cdm did not significantly increase EC networks. In contrast, bv-Cdm stimulated network formation by 40%. Angiogenesis array analysis of av-MSC-Cdm and bv-MSC-Cdm revealed a lower expression of angiogenic factors than EC-Cdm. ELISA analysis showed that EC produced PIGF, PDGF and FGF, and bv-MSC secreted VEGF, while av-MSC did not produce any of the stated growth factors.

**Conclusion**

In conclusion, both av-MSC and bv-MSC enhance endothelial cell viability via paracrine factors. For therapeutic treatment, pv-MSC might be valuable to stimulate angiogenesis in ischemic tissues, whereas av-MSC rather promote the survival and stabilization of blood vessels without the risk of unmeant angiogenesis.
Development of an In vitro Scaffold-based 3D Human Respiratory Tract Model

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To understand pulmonary disease mechanisms, it is vital to study the process of antigen entry and recognition by the immune system. We are developing a triculture model of the upper respiratory tract consisting of 3 key cell types – epithelial cells: the first line of defence against allergens and pathogens; fibroblasts: which provide extracellular matrix (ECM) proteins; and dendritic cells (DCs): professional antigen presenting cells that drive the adaptive immune response. In the upper respiratory tract, DCs sample antigens through the airway epithelium and respond to danger signals. We believe this model will allow the study of DC activation and the pulmonary innate immune responses in a physiologically relevant environment.

We have established co-cultures comprised of epithelial cells and fibroblasts supported on 3D electrospun polymer scaffolds that mimic the structure of lung ECM and support growth and differentiation of fibroblasts and epithelial cells. Here we present the successful establishment of a differentiated epithelial barrier expressing tight-junctions at the air-liquid interface on highly porous 3D scaffolds. Furthermore, we demonstrate that co-culture with fibroblasts promotes epithelial barrier formation and repair. We have also successfully incorporated monocyte-derived human DCs on 3D scaffolds and our current work focuses on their incorporation into the triculture model and assessment of functionality in this system. We propose that complete 3D model could efficiently mimic the structure and cellular environment of the upper respiratory tract and thereby provide an effective in vitro tool to investigate key respiratory diseases (e.g. asthma, COPD) and assess therapeutic agents.
We hypothesize that a controlled, inflammatory environment may have a pro-osteogenic effect on multipotent mesenchymal stromal cells (MSCs) in bone regeneration strategies. Our first aim is to identify inflammatory molecules capable of mediating direct effects on the osteogenic differentiation of MSCs in vitro. In this study, bone marrow-derived human MSCs, either uncommitted or pre-differentiated into pre-osteoblasts, were co-cultured with different doses of human recombinant TNF-α and LPS. As such, cells were cultured in growth medium or osteogenic differentiation medium containing BMP-2 or dexamethasone/L-ascorbic acid-2-phosphate. Alkaline phosphatase activity and calcium deposition were measured as early and late markers of osteogenic differentiation respectively. Results show that the pro-inflammatory mediators TNF-α and LPS have a direct, beneficial effect on the osteogenic differentiation of human MSCs compared to the controls. Importantly, this is only observed when an osteogenic stimulus is present, i.e. BMP-2 or dexamethasone. Moreover, these pro-osteogenic effects were similar in immature and differentiated pre-osteoblasts. Finally, differences in outcome were observed depending on the osteogenic stimulus used. Because the concurrent delivery of corticosteroids with inflammatory molecules in vitro often results in a discrepancy with observations in vivo, the use of BMP-2 as an osteogenic supplement comprises an alternative model.
Limited therapeutic options for the treatment of cartilage injury and degeneration have driven the development of tissue engineering and cell therapy alternatives. We have investigated the secretion of cartilage matrix-associated sulphated glycosaminoglycan (SGAG) species from primary human bone marrow mesenchymal stem cells (BMA13), chondrocytes (OK3) and embryonic stem cell derived cells (1C6) to characterise cellular chondrogenic potential. SGAGs are the sulphated polysaccharide units of cartilage proteoglycans and are responsible for maintaining tissue hydration. Cells were seeded in 2-D and chondrogenesis induced over twenty days using pro-chondrogenic media (PCM). SGAG content of the cell monolayer and media was quantified using the dimethyl methylene blue assay to determine total and secreted SGAG and then normalised to DNA level. In all cases larger quantities of SGAG were produced by cells in proliferation media than in PCM. OK3 secreted the greatest amount of SGAG (62.6 µg SGAG/µg DNA) while BMA13 secreted the least (31.4 µg/µg) where a substantial proportion of this, up to 98%, was detected in the media fraction. In the presence of PCM total SGAG secretion was reduced by approximately 2-fold in all cell types tested whereas the substrate associated fraction was significantly increased in both 1C6 (16% to 36%) and BMA13 (4% to 15%) (p<0.001 at day 20) when compared to non-PCM controls. In conclusion a pro-chondrogenic influence reduced total SGAG with a concomitant significant increase in the proportion of extracellular matrix-associated SGAG. Ongoing studies will extend these observations into micromass pellet systems and determine whether the SGAGs being produced are characteristic to those of articular cartilage.
Investigating Cell Seeding Techniques for Optimising Attachment of Human Mesenchymal Stem Cells on Polycaprolactone Electrospun Scaffolds

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When performing in vitro cell testing, the number of cells to be seeded is determined and administered to the substrate at a known concentration. Despite seeding a known number of cells, the actual number that attach cannot be assumed to be 100%, particularly when seeding cells onto scaffolds of electrospun fibres. We have investigated seeding of cells onto electrospun scaffolds that are held within different set-ups in order to determine the ideal seeding protocol. 3D electrospun polycaprolactone scaffolds were mounted into either custom-made PTFE troughs, 6-well CellCrowns (Scaffdex), or placed within Synthecon vessels (10 ml) rotating on a RCCS-4DQ bioreactor (9 RPM). PTFE troughs and CellCrowns were kept under static or shaken (30 RPM) conditions. Bone marrow derived hMSCs were seeded onto the scaffolds (20,000 cells) and cultured for 4 hours at 37°C, 5% CO₂. The number of cells seeded was based on the approximate surface area of the scaffold available for cell attachment. The location of cells was determined by PicoGreen DNA assay of scaffolds, media and well (n=4). Initial cell attachment was relatively low for all set-ups investigated (Fig.1), with the majority of cells located within the media and well. Cell attachment to the scaffold, however, was improved when shaken at 30 RPM. Further investigation at longer time-points, including 24 hrs, will be performed in order to optimise the ideal seeding set-up and attachment time.

Fig.1 DNA content (Mean ± Standard deviation) calculated for cells located on the scaffold, within the media and within the well, 4 hours post-seeding for each seeding set-up.
Harnessing the Potential of Stem/Progenitor Cells Coupled with Biomaterials for Human Muscle Tissue Engineering

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Muscle disorders encompass a wide range of acquired or inherited, acute or chronic diseases that affect skeletal muscle tissue with variable severity (e.g., traumatic lesions and muscular dystrophies). Regenerative cell-based therapies are entering clinical experimentation for muscular dystrophies and their combination with biomaterials holds great promises for tissue engineering. However, limited evidence is available on safety and efficacy of human muscle stem cells combined with biomaterials. Here we explore the capability of various biomaterials to sustain and improve viability, growth and differentiation of muscle stem/progenitor cells.

To this aim, we started evaluating the influence of the culture surface stiffness on mouse and human muscle stem/progenitor cell growth and myogenic differentiation. We showed that a higher stiffness (28 kPa) gives better results compared to lower ones (0.5 - 12 kPa) in standard culture/differentiation conditions. Moreover, we demonstrated that the combination of different clinically relevant biomaterials, namely compressed collagen hydrogels, polyethylene glycol (PEG)-fibrinogen hydrogels and poly-lactic co glycolic acid (PLGA) porous microspheres, is able to sustain cell adhesion, growth and myogenic differentiation. Importantly, all these systems can be modeled in 3-dimensional structures that help to resemble the physiological cell-cell interaction and organization of the muscle tissue.

Future perspectives of this work include tissue replacement protocols for local conditions and disease modeling platforms using patient-specific cells to test novel molecules able to counteract disease progression in systemic disorders.
Ectopic Liver Rescues Rats with Experimentally Induced Liver Fibrosis

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It has been shown previously that transplanted hepatocyte can engraft and survive in the several different extrahepatic sites (ES), including peritoneum, lung, pancreas, etc. Therefore, hepatocytes transplantation (HT) to ES has a potential for cell-based therapy. Data published by Hoppo et al. in 2011 demonstrated that mouse lymph node can support the engraftment and proliferation of hepatocytes as ES and rescue Fah mice from lethal liver failure. The aim of our study was to evaluate the allogeneic hepatocytes engraftment (HE) in albino rats with liver fibrosis (LF) induced by N-nitrosodimethylamine (NDMA). To achieve this goal, 10 mg/kg of NDMA was injected i.p. three times a week for 4 weeks (n=36). LF was confirmed by histological analysis of the liver samples. After NDMA exposure 18 rats were i.p. injected with 5×10⁸ freshly isolated hepatocytes; intact rats (n = 18) served as a control group. We observed 30% mortality rate among rats with LF within 1 week after NDMA exposure cessation, while 100% of animals with HT survived. Liver functions were assessed by quantifying blood biochemical parameters (ALT, AST, GGT, total protein, bilirubin and albumin) at 1 week, 1 month and 2 months after HT. We found that HT significantly normalized liver functions in experimental group. To confirm a hepatocytes' engraftment, immunohistochemistry of spleen, intestines, stomach and lungs has been conducted. We have found HepPar1 positive cells mainly within enlarged Peyer's patches (aggregated lymphoid nodules in the lowest portion of the small intestine). In conclusion, i.p. transplantation of hepatocytes improved animal survival and blood biochemistry by generating an ectopic hepatic mass inside the Peyer's patches.
Self-renewal of Human Embryonic Stem Cells on Synthetic Electrospun Nanofiber Substrates

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Human embryonic stem cells have great potential for therapeutic use in tissue engineering-driven regenerative medicine applications. This is in large part due to their pluripotent differentiation potential and relative ease of scale-up. Human embryonic stem cells (hESCs) are conventionally expanded and maintained in vitro on biological substrates. Synthetic substrates such as electrospun polymer nanofibers, present an opportunity to optimise xeno-free hESC culture regimes. In this study, three synthetic polymers were electrospun (aligned and random conformations) and seeded with hESCs. Clonogenicity experiments demonstrated that nanofiber scaffolds supported hESC adhesion and expansion only in physiological normoxia (2 % O2). Concomitantly, in hyperoxia (21 % O2), small, dense, and tightly packed, apparently adherent, embryoid body-like clusters were formed instead. In both instances, greatest number of colonies was observed on poly-ε-caprolactone (PCL) nanofibrous scaffolds. Furthermore, decreasing the fiber diameter of PCL nanofibrous scaffolds significantly increased hESC clonogenicity. Expression of pluripotent markers and retention of differentiation capacity was retained in hESCs cultured on PCL scaffolds. These results demonstrate the potential of nanofibers as xeno-free scaffolds supportive for hESC adhesion, expansion, differentiation, and also potentially as transplantable scaffolds.
Comparative Correlation Analysis of Sox9, 5, And 6 As Well as Col2a1 and Aggrecan Expression of Collagen I-Implant-Derived and Osteoarthritic Chondrocytes

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Matrix-assisted autologous chondrocyte implantation is frequently applied to replace damaged cartilage in order to support tissue regeneration or repair and to prevent progressive cartilage degradation and osteoarthritis. Its application, however, is limited to primary defects and contraindicated in the case of osteoarthritis which is partially substantiated to dedifferentiation and phenotype alterations of chondrocytes obtainable by patients' biopsies. The differentiation state of chondrocytes is reflected at the level of structural gene (Col2a1, aggrecan, Col1a1) and transcription factor (Sox9, 5, 6) expression.

Hence, we determined the mRNA abundances of Col2a1, aggrecan, and Col1a1 as well as Sox9, 5, and 6 of freshly isolated and passaged collagen I-implant-derived and osteoarthritic chondrocytes. Moreover, we analyzed the correlation of structural and transcription factor gene expression. Thus, we were able to evaluate the impact of the mRNA levels of transcription factors on the expression of cartilage-specific structural genes.

Significant differences were obtained for (1) freshly isolated osteoarthritic vs. collagen I-implant-derived chondrocytes, (2) due to passaging of the respective cell sources, (3) osteoarthritic vs. non-osteoarthritic chondrocytes, (4) Col2a1 vs. aggrecan expression with respect to the coherence with Sox9, 5, and 6 transcript levels.
In Vitro Tissue Regeneration by Human Degenerated Nucleus Pulposus Cells in Hyperosmotic Culture Medium

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Intervertebral disc (IVD) cells normally reside in a high osmolality environment of 450 to 550 mOsm/kg. In bovine healthy IVD cell culture, it has been shown that matrix production, cell proliferation and gene expression increase with osmolality, but it is not known how human degenerated disc cells are affected by osmolality, nor what osmolyte would be most effective. The aim of the current study was to determine the optimal culture conditions with respect to medium osmolality for human degenerated NP cells in terms of regeneration.

The osmolality of standard culture medium was adjusted with NaCl, urea and sucrose from 340 to 400, 450 and 500 mOsm/kg. IVD cells from two human donors (Thompson grade III) were cultured for 28 days in high density ($1\times10^6$ cells/cm$^2$) on collagen II coated filters. Matrix content as reflected by glycosaminoglycan (GAG) production was measured with a dimethylmethylene blue (DMMB) assay and DNA content with a Picogreen assay. Univariate analysis of variance with randomized block design and post-hoc Dunnet $t$-test were performed.

No effect was found on increasing osmolality using NaCl, whereas increasing osmolality with urea or sucrose resulted in significantly lower amounts of GAG per DNA ($p=0.05$ and $p<0.001$, respectively).

These results indicate that increasing osmolality does not affect the regenerative response of IVD cells, but using urea or sucrose to adjust osmolality inhibits GAG production. Effects on collagen deposition and gene expression are currently being evaluated. In conclusion, in terms of GAG production per DNA, it does not seem to be favorable to adjust osmolality and using urea or sucrose even decreases matrix production.
The Effect of Hypoxia on Endothelial and Chondrogenic Differentiation of Human Amniotic Fluid-Derived Stem Cells (hAFSCs) for Use in Orthopaedic Tissue Engineering

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hAFSCs represent a promising stem cell source for tissue engineering due to their pluripotentiality and high renewal capacity. This study seeks to engineer, in vitro, therapeutic constructs for bone and cartilage repair using hAFSCs in combination with collagen-GAG scaffolds from our lab. We propose that exposure of cell-seeded scaffolds to hypoxic conditions (activating the Hypoxia Inducible Factor-1 pathway) might create an environment more appropriate for de novo vascular or cartilage tissue formation within the construct. In order to investigate the effect of hypoxic conditions on endothelial and chondrogenic differentiation, hAFSCs were cultured in 2% O₂ on either collagen-chondroitin sulphate (coll-CS) scaffolds for endothelial differentiation, or on collagen-hyaluronic acid (coll-HyA) scaffolds for chondrogenic differentiation.

hAFSCs differentiated in hypoxia presented an endothelial gene expression profile more closely resembling that of endothelial cells than hAFSCs differentiated in normoxia. This was paralleled by enhanced VEGF protein production and increased tubule formation, indicating the beneficial role of hypoxia in endothelial differentiation. Moreover, chondrogenesis of hAFSCs seeded on coll-HyA scaffolds was enhanced when cultured in hypoxia, as evidenced by increased sGAG production as well as accelerated expression of early chondrogenic genes (e.g. Collagen II). Cartilage matrix deposition within the scaffold was also significantly enhanced.

The results of this project indicate the suitability of hAFSCs for use in a number of areas of regenerative medicine as well as illustrating potential of utilising a low-oxygen environment to enhance the development of engineered constructs.
Tissue-mimetic Matrices as Cell-instructive Microenvironments

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Deciphering the role of extracellular matrices (ECM) in stem cell niches is hampered by the lack of suitable methods for recapitulating complex ECM microenvironments \textit{in vitro}. Therefore, we have introduced a protocol that permits reliable anchorage of native cell-secreted ECM to culture carriers. The approach is validated by two distinct types of human bone marrow (BM) specific decellularized ECM substrates that were demonstrated to support human mesenchymal (MSC) and hematopoietic stem and progenitor cells (HSPC) \textit{in vitro}. The MSC-derived ECM preparations were thoroughly characterized with respect to composition and structure. We report the unique ability of these complex ECMS to support expansion and differentiation of BM stem cells. The established methodology enables modulating native-like multicomponent ECMS of tissue-resident stem cells, and can therefore be expected to allow for a more rational design of engineered stem cell niches.
As the field of regenerative medicine and stem cell engineering started to play important role in the new generation of bioengineering, temporal and spatial encapsulation of the cells by the hydrogels are getting an attention as a novel way to handle cells in the three dimensional conditions. Understanding the relationship between cell function and its environment is important to take passive control over the cell function through outer signals. Polymeric cellular environment is full of potentials for these needs since the environmental properties could be constructed. Bioinspired polymer, poly(MPC-co-n-butyl methacrylate-co-p-vinylphenylboronic acid) (PMBV) /poly(vinyl alcohol)(PVA) hydrogel matrix were prepared spontaneously by mixing of the two polymers in a cell culture medium. The storage modulus of the PMBV/PVA hydrogel matrix was controlled from 0.3 kPa to 2.5 kPa that corresponded to very soft natural tissue. The proliferation ratio of non-oseteogenic mouse pluripotent cells, C3H10T1/2 was influenced by the storage modulus of the PMBV/PVA hydrogel matrix. When the storage modulus of The PMBV/PVA hydrogel were above 1.0 kPa, the proliferation ratio was suppressed and provided uniformed cells in G1-phase. High G1-phase fraction will lead to excellent differentiation efficiency, which will lead to a great insight to the stem cell engineering field.
Capillary-Like Structure and Vascular Lumen Formation Generated By Different Supportive Cell Types in a Tri-Culture System

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Introduction: For a tissue engineered full-thickness skin equivalent, a blood vessel system with complete lumen formation is required to support the surrounding cells with oxygen and nutrients. To ensure their supply, the control of capillary lumen formation is necessary. In co-culture systems, lumen formation in capillary-like structures can already be observed, although a complete lumen could not be realized by skin fibroblasts alone. Therefore the potential of normal human foreskin keratinocytes (NHFKs) supporting lumen formation was investigated.

Experimental: As a supportive cell layer, human dermal foreskin fibroblasts (HDFFs) were seeded on well-plates followed by human umbilical vein endothelial cells (HUVECs). Fibrin gels were cast on top of the co-culture system. To enhance the adhesion and support of NHFKs in different concentrations, fibronectin or fibroblasts have been applied in or on top of the fibrin gel. NHFKs were subsequently seeded on top of the fibrin gel. After 14 and 21 days of tri-culture, fibrin gels were fixed and immunostained (CD31). Verification and quantification of the luminal segments in capillary-like structures were implemented by two-photon laser scanning microscopy (TPLSM) and ImagePro Analyzer software.

Results: After 14 and 21 days of tri-culture, it could be observed that NHFKs significantly improved the endothelial lumen formation in vitro compared to the HDFF/HUVEC co-culture system alone. Lumen formation was dependent on both NHFK cell number and donor.

Conclusion: Endothelial lumen formation in a 3D tri-culture system can be improved by NHFKs compared to the conventional HDFF/HUVEC co-culture system. The amount of lumen formation is dependent on cell number and donor.
Cartilage extracellular matrix (ECM) derived scaffolds have shown great promise for cartilage regeneration. Combining Mesenchymal stem cells (MSC) with such scaffold represents a promising approach to engineer functional cartilage grafts, although the scaffold characteristics to realise such aim remain to be elucidated. Previous work shows that the morphology of a scaffold regulates MSC proliferation and differentiation. In this study it was investigated the influence of ECM concentration and scaffold architecture on the migration and differentiation of diseased human infrapatellar fat pad derived SC (FPSC) seeded in ECM derived scaffolds. To modulate the architecture of the scaffolds, porcine articular cartilage was processed to produce slurries consisting of either ‘coarse’ or a ‘fine’ ECM particles. Slurries with different concentrations of ECM (250, 500, 1000mg/ml) were produced. He ion microscopy revealed a larger pore size and a more heterogeneous structure in the ‘coarse’ scaffold when compared with the ‘fine’. ECM concentration influenced MSC migration and distribution throughout the scaffold, with a more homogenous distribution of cells observed in lower concentration, especially for ‘fine’ 250mg ECM scaffold, indicating that MSC could more easily propagate through these constructs. Overall, sGAG accumulation within MSC seeded constructs was greater in the higher concentration ‘coarse’ scaffolds. However, as the concentration of ECM within the scaffolds was reduced, superior chondrogenesis was observed in the ‘fine’ scaffolds. The results of this study demonstrate that both the concentration of ECM in the scaffold, and their structure, regulate the migration and chondrogenic differentiation of human FPSC.
Determining Optimal Cell Ratios of Nucleus Pulposus and Mesenchymal Stem Cells Co-Cultured in a Nutrient Deprived Microenvironment

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Mesenchymal stem cell (MSC) strategies have been proposed for the treatment of intervertebral disc (IVD) degeneration. However, a key factor for clinical translation is understanding how MSCs will perform in the harsh microenvironment of the IVD. This study investigated the performance of bone marrow (BM) MSCs cultured with nucleus pulposus (NP) cells at different cell ratios in a nutrient-limited in vitro model using radial confinement to recapitulate the predominant in vivo nutrient diffusion route of the cartilage endplate (CEP).

Porcine NP and BM cells were suspended in 2% agarose at different NP:BM ratios (1:1, 8x10^6 cells/ml and 1:2, 12x10^6 and 1:4, 20x10^6 cells/ml). Confined constructs (diameter:5mm, height:3mm) were inserted into silicone chambers to confine the radial and base surfaces whilst unconfined constructs were cultured in free swelling conditions. Constructs were maintained under IVD-like conditions (low-glucose, low (5%) oxygen) with TGF-β3 supplementation for 21 days.

Results showed that cell viability diminished with increasing cell density for both confined and unconfined conditions. sGAG content (%w/w) was higher for unconfined constructs while collagen content (%w/w) was higher for confined constructs. Overall, a cell ratio of 1:1 performed best in terms of maintaining cell viability and promoting matrix accumulation. The confined in vitro model developed in this work provides a useful system in determining optimal cell ratios for clinical translation as it more closely resembles the constraints of nutrient and oxygen supply within the disc which may have important implications for cell based strategies to regenerate the IVD.
Influence of Substrate Stiffness on Macrophage Behavior

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During inflammatory processes, such as fibrosis and the foreign body reaction, macrophages encounter relatively stiff matrices ranging from 100 kPa in fibrotic environments to several orders of GPa on implanted biomaterials. Since macrophages sense and respond to changes in the mechanical properties of their extracellular environment, we question how matrix stiffness affects macrophage behavior in terms of their polarization capacity and their capacity to form giant cells. Murine macrophages were seeded on polyacrylamide gels with an elastic modulus ranging from 6 kPa to 105 kPa and stimulated with LPS/IFNγ or IL4/dexamethasone to induce polarization. Gene expression analysis showed that the stiffness of fibrotic tissue (105 kPa) increased the polarization capacity to both M1 and M2 phenotypes. Giant cell formation was induced by prolonged stimulation with LPS. On gels with a stiffness mimicking fibrotic tissue, giant cell formation was increased. Taken together, our data indicates that the stiffness of the matrix encountered by macrophages during fibrosis and the foreign body reaction may favor their polarization and their ability to form giant cells.
Human Umbilical Vein Endothelial Cells (HUVECs) Vascular Morphogenesis on Cell-derived Extracellular Matrix

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Extracellular matrix (ECM) is a complex of diverse macromolecules that provides cell binding motifs, structural support, and signaling cues for cell adhesion, proliferation, and differentiation. We hypothesize that cell-derived extracellular matrices (CD-ECM) possess unique surface texture and compositions, and therefore can provide distinct signals for vascular morphogenesis of HUVECs. CD-ECMs were directly obtained by in vitro-cultured fibroblast, preosteoblast, or chondrocyte and they were named FDM, PDM, and CHDM, respectively. A significant difference of surface morphology, matrix thickness and roughness were observed among the CD-ECMs. HUVECs proliferation on CD-ECMs was comparable with positive control. A capillary-like structure (CLS) formation of HUVECs was obvious with PDM and FDM after 5-day in vitro culture, whereas no CLS was found with CHDM and gelatin. Furthermore, quantitative analysis of capillary branch point and branch length also exhibited a significant improvement of such parameters with PDM and FDM. The average thickness of a new capillary was about 10 μm, as determined by confocal microscopy. In addition MT1-MMP and MMP-1 were highly expressed with PDM, suggesting the matrix remodeling for the vascular morphogenesis of HUVECs on 2D environment. This study indicates that each CD-ECM carrying unique physiochemical features holds a significant impact in triggering or inhibiting angiogenic activity of HUVECs.
Healing of cartilage defects is an important medical problem since the current treatment methods are ineffective to restore full function and return the tissue to its healthy state. Having a slower metabolism than other tissues, cartilage tissue cannot fully repair itself after damage. For this reason, developing therapies for the treatment of cartilage tissue damages that occur as a result of common joint diseases like osteoarthritis, rheumatoid arthritis and accidents, is of major importance. Regeneration of damaged cartilage tissue and complete recovery of its functionality may be possible with regenerative medicine studies, which hold great promise by offering novel solutions for generation of functional tissue substitutes. Glycosaminoglycan (GAG) molecules are important constituents of both developing and mature cartilage extracellular matrix (ECM). Several studies indicate that actions of regulator proteins of cartilage development depend on these GAGs. In this study, we explored the role of GAG mimetic self-assembling nanofibers as a scaffold in inducing chondrogenic differentiation of chondroprogenitor ATDC5 cells and mouse mesenchymal stem cells. Chondrogenic differentiation was indexed by sulfated GAG deposition and expression of cartilaginous ECM proteins such as collagen II and aggrecan. Moreover, expressions of genes specific to cartilage tissue were investigated to reveal the effect of GAG mimetic peptide nanofibers on chondrogenic differentiation.
A defining characteristic of tendon cells is their elongated morphology within a matrix of highly organized collagen fibrils. When removed from their native environment, tendon cells lose their characteristic morphology and expression of phenotypic markers changes. Within this study, the ability of scaffold architecture to maintain tendon cell shape and phenotype was evaluated in vitro, in the presence and absence of a supporting fibrin gel. Scaffolds with axial and isotropic pores were seeded with adult, ovine, tendon derived cells, with and without fibrin gel. Cells could proliferate on all scaffold types but exhibited morphological differences. Cells appeared more elongated when cultured on axial scaffolds in all culture conditions. The highest proliferation and metabolic activity were observed on scaffolds with fibrin gel, regardless of architecture. However, protein expression varied with scaffold orientation. Axial scaffolds promoted increased tenomodulin production: a marker for mature tendon cells, and decreased scleraxis expression: an early stage transcription factor for tendon cell lineage. Incorporation of fibrin gel increased the expression levels of tendon markers, but the trends in protein expression with scaffold orientation remained the same. This study suggests that cell morphology, promoted by scaffold architecture, is important in maintaining a mature tendon cell lineage in vitro.
Macrophage-mediated Upregulation of VEGF in Co-cultures of Out growth Endothelial Cells with Primary Osteoblasts

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Inflamatory cells like macrophages (MΦ) or monocytes (MNC) secrete proinflammatory cytokines and proangiogenic factors, finally contributing to the formation of new blood vessels. The co-culture system of outgrowth endothelial cells (OEC) and primary osteoblasts (pOB) represents a model to evaluate the different modulatory activity that MΦ or MNC may have. The aim of this study was to investigate if MΦ or MNC might positively influence the formation of a microvasculature via inflammatory processes in the co-culture system. The monocytic cell line THP-1 was fully differentiated into the MΦ phenotype and added to the co-culture consisting of OEC and pOB. Co- and triple-cultures were cultivated for 7/14d before they were analyzed for inflammatory signals and angiogenesis activation using immunohistochemistry, ELISA and real-time RT-PCR.

The treatment of co-cultures with MΦ or MNC resulted in an upregulation of inflammatory cytokines (IL-6 and IL-8) and adhesion molecules (E-Selectin and ICAM) after 7d, evaluated by RT-PCR. The proangiogenic factor VEGF was also upregulated after both time points in co-cultures containing MΦ or MNC as evaluated by ELISA and RT-PCR. Immunofluorescent staining of triple-cultures exhibited a higher amount of microvessel-like structures at both time points than in the absence of the inflammatory cells. Addition of MΦ or MNC to co-cultures seems to induce the inflammatory process with ensuing acceleration of formation of microvessel-like structures.
Cryopreservation of adipose-derived stem cells using polycaprolactone (PCL) scaffold

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Cell-related technologies, such as cellular therapeutics are gaining interest and are used in the treatment of several diseases. Furthermore, clinical researches using stem cells are underway for the various indications. However, the sensitivity of cells toward freezing and thawing which induces lower survival rate and changes of cellular characteristics limits the development and application of cell therapies. Therefore effective and safe cryopreservation method which could yield better survival rate of cells is required and here we reports cryopreservation method of adipose-derived stem cell using a scaffold.

We prepared polycaprolactone (PCL) scaffolds with free-form fabrication machine. Adipose-derived stem cells are seeded on the scaffolds and attached overnight in CO₂ incubator. These stem cells attached to the scaffold were frozen with the addition of cryoprotectants and control cells which were not attached to the scaffold were frozen with the same protocol. After thawing, we compared the survival rate, adhesion rate and growth rate of both group. The cells attached to scaffold showed significantly higher survival rate, cell adhesion rate and growth rate than the control group.

From these results, the use of a scaffold for the cryopreservation of cells is considered to be suitable and more results are to be followed.
Osteoarthritis (OA) is a degenerative disease characterized by the degradation of articular cartilage. Human bone marrow mesenchymal stem cells (BM-MSCs) constitute an attractive source for cartilage tissue engineering however depend on the development of efficient and controlled chondrogenic methods. This study is aimed at analyzing the chondrogenic potential of BM-MSCs, cultured using a conventional method and a three-dimensional system (PEPMHA) where the presence of hyaluronic acid could provide a favourable niche for MSCs chondrogenesis, under hypoxia or normoxia environments.

BM-MSCs isolated from OA or N patients cultured either allowing spontaneous spheroid formation or on PEPMHA hydrogels, were chondrogenically induced, under hypoxia or normoxia (5 or 21% pO2) for 7, 14 and 21 days. Glucose and lactate dehydrogenase were measured. Histological analysis and the molecular profile of cells was performed using RT-PCR and the relative expression of collagen types I, II and X, SOX9, aggrecan, MMP13, Runx1 and 2 were evaluated.

Chondrogenic differentiation of BM-MSC was observed in the different conditions evaluated. An upregulation of chondrogenic markers was obtained when cells were seeded in PEPMHA hydrogels and submitted to 5% pO2 that simulates the physiological oxygen tension in cartilaginous regions. This condition also elicited a downregulation of hypertrophic markers.

**Conclusion**

MSCs chondrogenic commitment can be improved by the election of appropriate culture systems and biophysical stimuli. We hereby show the benefits of using a 3D hydrogel combined with hypoxia in the enhancement of the chondrogenic potential of MSC and the delay of their terminal differentiation. Fig. 1: Collagen type-II gene expression, obtained by real-time PCR, of BM-MSC (OA) differentiated in normoxia versus hypoxia conditions and under spontaneous spheroid formation versus hydrogel culture, after 21 days.
Evaluation Of Proliferative Effect Of Low-Level Laser Irradiation On Bone Marrow-Derived Mesenchymal Stem Cells: An In Vitro Analysis.

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Several studies have shown that low-level laser irradiation (LLLI) promotes proliferation in many cell types, but the exact molecular and cellular mechanisms involved in this process remain poorly understood. Human bone marrow-derived mesenchymal stem cells (hBMSCs) have shown to be an appealing source for cell therapy and tissue engineering. In this study we examined the in vitro effect of a single or a multiple doses of LLLI on proliferation of MSCs isolated from adult human bone marrow of different patients. The cells were irradiated with a red wavelength diode laser (659 nm) and exposed to different laser doses (0.5, 2, 5 J/cm²) for 1 day and 3 consecutive days. Proliferation was evaluated using a quantitative assay. Both single and multiple doses of LLLI enhanced the proliferation of hBMSC isolated from all patients. A significantly cell growth increase was detected in groups irradiated for three consecutive days compared to a single dose or control group. However, differences was observed between the proliferation rates of hBMSC from different patients. This preliminary results showed that LLLI affected positively hBMSCs proliferation in vitro and it may have an important impact for the use of mesenchymal stem cells in regenerative medicine: in fact, LLLI may be use as a “photoceutical” bioreactor for in vitro stem cells preconditioning prior to transplantation. As a future perspective, we would like to investigate the potential of LLLI for promoting regeneration also for stem cells cultured on biomaterials.
Pro-pathologic Evolution of Human Aortic Valve Interstitial Cells Phenotype is Induced by Substrate Stiffness

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Introduction Aortic valve (AoV) stenosis is a relevant disease, due to aortic valve interstitial cells (aVICs) conversion into myofibroblasts or bone-depositing cells. In the present study, we aimed at investigating the role of substrate stiffness in human aVICs differentiation into pathologic phenotypes.

Polyacrylamide gels (PAAg) of defined stiffness were designed and produced adjusting acrylamide concentration and Acryl/Bis-Acrylamide ratio. After Collagen I functionalization, PAAg surface stiffness was measured by atomic force microscope (AFM). Primary human aVICs were seeded onto PAAg and cultured for 48hrs followed by fixation and immunofluorescence staining.

Results of aVICs culture onto Collagen I-coated PAAg of four increasing stiffness, showed cell attachment and survival. Cell morphology was strikingly affected, with a more spread shape on higher stiffness substrates. In addition, the cytoskeleton organization showed an increase in polymerized actin fibers onto stiffer gels, suggesting conversion of these cells into myofibroblasts, cells typically participating in AoV stenosis. These results suggest that the response of human aVICs to increasing stiffness modifies their phenotype into that of pro-inflammatory cells. This establishes, for the first time, a direct relationship between mechanical compliance of the human AoV tissue and pro-pathologic evolution of valve resident progenitors.
Mesenchymal stem cells (MSCs) isolated from bone marrow (BMSCs) or adipose tissue (ASCs) have been deeply characterized for their usefulness in musculoskeletal tissue regeneration. However, other potentially valuable MSCs sources have been recently indicated. The goal of this study was to isolate MSCs from human tendons (TSPCs) and to compare their features with that of human ASCs.

Human TSPCs and ASCs were isolated from ST/G tendon portions and adipose tissue of healthy donors undergoing ACL reconstruction (n=6) or liposuction (n=6). At passage 1 (P1) the number of TSPCs per gram of tissue was significantly higher than ASCs (p<.001) and, at P3, TSPCs grow faster than ASCs, with a significantly lower doubling time (p<.05). Both population showed a great and similar clonogenic ability (CFU-F assay) and were positive for the typical MSCs surface antigens, such as CD90, CD105, CD73 and CD44 (FACS analysis). Both osteo-differentiated TSPCs and ASCs showed a significant increase of ALP activity levels (p<.05) respect to CTRL, but only ASCs were able to produce a significant amount of calcified matrix respect to CTRL (ARS staining quantification). Adipogenic differentiation ability of TSPCs was significantly lower than ASCs (ORO staining quantification), whereas only TSPCs, and not ASCs, showed a significant increase in GAGs production respect to CTRL (p<.05) when cultures in chondrogenic medium.

In conclusion, TSPCs possess most of the specific features which characterized mesenchymal stem cells, including the immunophenotype and the osteogenic potential, together with a pronounced chondrogenic ability. Our data show that tendon tissue could be a reliable source of MSCs for musculoskeletal tissue engineering application.
Hematopoietic stem cell transplantation (HSCT) is a therapeutic approach in treatment of hematological malignancies and incompatibility of Bone marrow. Umbilical cord blood (UCB) known as an alternative for hematopoietic stem/progenitor cells (HPSC) for allogenic transplantation. The main obstacle in application of HPSC derived from umbilical cord blood is the low volume of samples. So, ex vivo expansion of HPSCs is the useful approach to overcome this hinderence. The goal of using this systems is to produce appropriate amount of hematopoietic stem cells, which have the ability of transplantation and long term hematopoiesis. The conventional system of hematopoietic stem cells expansion is using cytokine coaktail in 2-Dimensional (2D) microenvironment. In 2D culture system cells only affected by cytokines and there is not any influence of cell – matrix intractions, migration and attachment. But 3- Dimensional (3D) microenvironments mimickes hematopoietic niche. Synthetic biomaterials such as microwells based on PDMS coated with Extracellular matrix (ECM) proteins is used to produce synthetic niches. SDF-1 is a member of CXC Family which attach to its receptor, CXCR4. SDF-1/CXCR4 complex plays critical role in homing of hematopoietic cells. This complex plays key role in transplantation of hematopoietic cells. These 3D structures, chemical and mechanical properties of these microwells leads to activation of adhesion, proliferation, differentiation and migration of CD133+ cells with highly similarity to ECM. In these microwells analouge materials of ECM or natural proteins such as collagen, fibronectin and laminin attachment and proliferation and expression of homing agents such as CXCR4.

Materials: In current study CD133+ cells were isolated from umbilical cord blood by MACS method. Isolated cells were seeded on microwells which were prepared by softlitography method. Expression of CXCR4 were analyzed by quantitative real time PCR on day 7 and 14 in 2D and 3D microenvironment.

Results: Our results showed that expression level of CXCR4 as a homing factor were increased in CD133+ derived umbilical cord blood in cells cultured in microwells compared to 2D microenvironment in day 7 and 14.

Conclusion: Our findings demonstrated that preparing a microenvironment which mimicks stem cell niche would help us to expand and proliferate hematopoietic stem cell derived umbilical cord blood in a condition which is more proper for stem cell transplantation.

**Keywords:** Umbilical cord blood, 3D environment, CXCR4, Homing
Chitosan/Organoclay Composite Scaffolds for Bone Tissue Engineering

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Introduction. Polymer scaffolds composited with organoclay (OC) have started to receive interest for bone tissue engineering applications. In this study, we have developed a scaffold composed of chitosan (C) and OC, and tested its ectopic bone-forming potential. Materials and Methods. OC was formed by cation exchange reaction between calcium smectite and anilinium chloride. The characterised organoclay dispersion was suspended in acidic chitosan solution, and the 3D scaffold was produced by freeze-drying. SEM, FTIR and XRD were applied to evaluate C/OC composite material. Then, MC3T3-E1 cells were cultured, expanded and seeded on C/OC scaffolds. MTT assay was used to evaluate MC3T3-E1 viability and metabolic activity; SEM demonstrated cell proliferation and morphology in vitro. Histocompatibility and ectopic bone formation potential of MC3T3-E1-seeded C/OC constructs was evaluated by transplanting them into epigastric fasciovascular flaps of Wistar rats. Explants were assessed by H&E and von Kossa stainings at 4 weeks-postoperatively. Results and Conclusion. FTIR and XRD results showed that C was successfully incorporated between organoclay layers. SEM images revealed that the scaffold was highly porous and had a rough surface morphology. Histochemical findings revealed that the constructs were well-tolerated by the subjects (H&E) and provided a suitable environment for new bone formation (von Kossa). Findings suggest that C/OC scaffold supports in vitro proliferation of MC3T3-E1 cells, presents high tissue compatibility and promotes the formation of calcified tissue matrix in vivo, pointing out the potential of preosteoblast-seeded C/OC composite system for bone regeneration applications.
Keratinized Epithelial Differentiation Capability of Human Umbilical Cord Cells as Determined by Immunohistochemistry Analysis

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Introduction: Human Wharton’s jelly stem cells (HWJSC) have inherent differentiation capabilities as compared with other adult mesenchymal cells, showing high expression of typical markers of undifferentiated cells such as SOX2, Oct4, CD90 and CD105. However, the epithelial differentiation capability of umbilical cord cells has been only attributed to epithelial amnioblasts. In this study, we analyzed several epithelial markers in umbilical cord tissues and isolated HWJSC in order to understand the epithelial differentiation capability of umbilical cord-derived mesenchymal cells.

Materials and Methods: Formalin-fixed, paraffin-embedded sections of the human umbilical cord and HWJSC primary cell cultures were obtained. Then, immunohistochemical analyses were carried out for some markers of non-keratinized epithelia (CK7, CK8, CK13) and markers of mature, keratinized epithelia (involucrin) in both the umbilical cord and the cultured cells. Human oral fibroblasts were used as controls.

Results and Conclusions: Our results showed that the protein expression of all these markers was very similar in umbilical cord tissues and cultured HWJSC. In addition, the expression of markers of keratinized epithelia was more strongly positive than markers of non-keratinized epithelia such as CK13. These results suggest that human umbilical cord-derived mesenchymal cells could have intrinsic epithelial differentiation potential, especially to keratinized epithelial types like skin keratinocytes.

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A computational Model to Assess the Contribution of Growth Factors to Phenotype Stability in Chondrocytes

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Cell-based tissue engineering constructs are an interesting expansion of the surgeon’s toolkit in treating long bone defects. However, the outcome of interventions with these constructs suffers from high variability barring their regular appearance in the clinic, in no small part due to the inter-patient variability in cell behaviour. In the paradigm of ‘developmental engineering’ a solution to this problem is envisioned by mimicking robust developmental processes in combination with a rigorous analysis thereof through the construction of computational models. From our knowledge of developmental biology we can form a computational model to facilitate understanding of how growth factors and transcription factors influence cell fate decisions in the growth plate and consequently answer the question whether – and how – they can boost bone healing.

The model presented in this study includes 46 factors and 146 interactions between them. The dynamics of the system were simulated in a simplified manner that differentiates between slow and fast interactions. Through a Monte Carlo approach the importance of each factor in the stability of chondrocytic phenotypes (proliferating, hypertrophic) is assessed. The hypertrophic state was found to be more stable than that of the proliferating chondrocyte. This higher stability in random initial conditions seems to be conferred by faster reactions that favor the hypertrophic phenotype. Overall, the model allows the importance of several important factors in the fate decision of chondrocytes to be quantitatively assessed and can make suggestions as to how an in vitro bone forming process could be steered.
The Role of Stem Cell-like Cells on Tubulogenesis Revealed by Micropatterning

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In the recent years, different studies have established a link between adult mesenchymal stem cells (MSCs) and perivascular cells like pericytes that envelop the vascular tubes. This link was validated mainly through shared marker signatures and raised the hypothesis that all MSCs are pericytes. A controversy however remains on whether there are functions specific only for one of the cell types. Here, we report the specific role of pericytes in vascular tube stabilization that is not shared with MSCs. An experimental model was introduced for the induction of endothelial cell (EC) tubulogenesis after 24 hours of incubation on micropatterned polymer surfaces. Pericytes or MSCs were added separately to this system in order to evaluate their effect the tubular stabilization. In the absence of additional cells or in the presence only of MSCs, the tubular structures were lost after 36h. Adding only pericytes however stabilized the EC vasculogenic tubes. Furthermore, only pericytes, but not MSCs, were able to migrate through a mimetic basement membrane and could interact with the ECs to stabilize the lumen structures. By demonstrating the multipotency of pericytes and their role on EC tubular structures stabilization, they can play a greater role in vascular and bone tissue regeneration. In the future, the use of these cells in tissue engineering becomes critical.

Background: Mesenchymal stem cells (MSC) are self-renewing multipotent cells which have the ability to differentiate at least into osteoblasts, chondroblasts and adipocytes. Hence they represent an immense potential in clinical applications. Due to the fact that MSC collection out of bone marrow (BM) is an invasive and very painful procedure, other tissues such as umbilical cord (hUC) have been considered. As far as cultivation of MSC is concerned fetal bovine serum (FBS) is still the most commonly used additive although it comprises many risks. In the course of this work we compared human serum (huS) and human platelet lysate (hPL) as alternative media supplements.

Methods: To isolate MSC, 6 hUC were obtained after caesarian sections. The cords were washed thoroughly with phosphate-buffered saline (PBS), minced into pieces of 1-3 mm³ and incubated in Petri dishes until a sufficient amount of MSC was adherent to the plastic surface. Cells were harvested and cultivated over three passages in T75 flasks (Sarstedt, Germany). Minimum essential medium alpha modifications (α-MEM) (PAA Laboratories, Austria) supplemented either with 5% hPL oder 15% huS was used. Both supplements were prepared in-house from whole blood donations with declaration of consent. The UC-pieces were incubated a second time for equal purpose under same conditions. Regular cell counts and flow cytometric analysis were carried out.

Results: The cells showed satisfying proliferation using both supplements. Proliferation of cells cultivated in hPL was slightly better although huS was added in threefold concentration compared to hPL. Cell yields harvested from cord pieces which were incubated a second time showed no significant decrease. Data on proliferation capacity and marker expression will be given.

Conclusion: Collection of MSC harvested from UCs by explant cultures offer an easy as well as cheap procedure to gain stem cells. Both hPL and huS are very promising substitutes for FBS. Because of their human origin they are the safer choice for MSC intended for future clinical use.
Characterization of Stem Cells from Equine Dental Pulp

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Dental pulp stem cells (DPSC) from mice and humans, expanded in vitro, show rapid proliferation and differentiation potential. Mononuclear cells from dental pulp of the molars of young horses (2-6 years old) were isolated by enzymatic digestion and cultured in Alpha-MEM or DMEM F/12 medium, supplemented with Fetal Bovine Serum (FBS) and 1% antibiotics and antifungicides. Adherent cells were analyzed by flow cytometry and immunocytochemistry for expression of markers of stemness (CD 90+, CD166+, CD34-, CD45-) and expression of embryonic stem cell markers (Oct 3/4, leukemia inhibitory factor receptor, Alpha-fetoprotein). Cultured cells expressed markers of mesenchymal stem cells and with the passages, the hematopoietic markers decreased. Cultures could be expanded and maintained in an undifferentiated state even after being frozen for up to six passages. Marker expression was similar to that identified in DPSC from other species in which differentiation assays have been successful for induction into several tissues in vitro. Now identified as equine DPSC (eqDPSC) for their characteristics as uncompromised cells, trials are needed to assess the plasticity and compare the potential to differentiate into various cell lineages when exposed to various growth factors. EqDPSC are a possible source of stem cells for their use in equine regenerative medicine.
Silated Chitosan and HPMC for Self-Setting Hydrogel For Tissue Engineering

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Tissue engineering is a multidisciplinary field combining chemical, engineering and life science expertises in order to develop functional structures for damage tissues. Cells are generally seeded into the synthetic extracellular matrix (SECM). These biomaterials are prepared as a temporary scaffold to improve mass transfer, support cell growth and enhance the cells to produce molecules they are supposed to. Until recently, it was believed that biomaterial selection was performed based on its macroporous, biocompatibility and degradability properties and its ability to withstand biomechanical stress. Engineering and material issues are now under focus to develop suitable scaffolds for tissue engineering. Indeed, Discher et al. have shown that material stiffness induces critical effects on cell behavior and differentiation. Therefore, LIOAD has been working on the development of hydrogels as SECM for tissue engineering. They prepared polymers capable of self cross-linkage (Si-HPMC, Si-Chitosan) to avoid any toxicity issues resulting from using cross-linking chemicals or photo cross-linking. The mixed Si-Chitosan/Si-HPMC hydrogels have shown good ability to control key physico-chemical properties such as viscosity, injectability, gel point and stiffness (tunable increase >10 times by adding Si-Chitosan). These networks have then been investigated for stem cell behaviour in 3D (viability, growth, differentiation, adhesion, migration) and good viability are been demonstrated.
In Vitro Evaluation of Novel PHBV-based Microfibrous Scaffolds Fabricated for Bone Tissue Engineering

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Poly(3-hydroxybutyrate-co-3-hydroxyvalerate), PHBV, is a thermoplastic polyester, which exhibits very good mechanical properties and piezoelectricity, therefore is of interest for application in tissue engineering of bone. Fused deposition modeling (FDM) enables precise control of scaffolds architecture. However, application of PHBV in the FDM process is very difficult due to rheological properties of the polymer. The aim of present study was to develop a novel PHBV-based material to fabricate 3D scaffolds by means of FDM and characterize their degradation profile, mechanical properties and bioacceptance. Composite scaffolds containing PHBV, poly(l-lactide-co-glycolide) and tricalcium phosphate nanoparticles were prepared by Bioscaffolder. The scaffolds were degraded in PBS solution for period of 3 months in dynamic conditions. During that time changes of mass, pH, porosity (µCT) and compressive mechanical properties were monitored. The bioacceptance of the scaffolds was studied with human Mesenchymal Stem Cells (hMSC) in form of multicellular spheroids (cell outgrowth, alkaline phosphatase activity and mineralization of extracellular matrix). Our modification resulted in 4-fold reduction of melt flow index of PHBV, which allowed for successful fabrication of scaffolds with expanded micromorphology of fibers. The scaffolds exhibited initial mechanical properties (E=220±40 MPa; σc=14±2 MPa) similar to those of cancellous bone, which kept increasing during first months of degradation. Mass loss at the end of experiment was of 1.2%. Preliminary bioacceptance studies indicated that the ternary composite scaffolds supported hMSC outgrowth from the spheroids and differentiation towards osteogenic lineage.
Amphiphilic Effect of Silicone-Modified Polysaccharide Molecular Hybrid on the Behavior of Human Skin Fibroblasts Applied for Wound Bed Preparation

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Although Polymer substrates have been revealed to be highly attractive materials such as implanted organ replacements, sensors, devices, and bioadhesive, in most biomedical applications they are hydrogels, especially intelligent hydrogels, whose polymer chains are strongly sensitive to large physical conformation changes in environmental stimuli and will exhibit much more complex responses to the surroundings. Due to the effect of conformational change, the cell-substrate interactions that occur over a small scale, such as physical, chemical, or biochemical, will become significantly difficult to control. Thus, here we address a new type of covalent amphiphilic polymer networks (CAPNs), i.e., PDMS-crosslinked-NOCC amphiphilic polymer networks (PMSC), by esterification between cross-link PDMS diol [bis(hydroxyalkyl) terminated polydimethylsiloxane, silicone] and NOCC (N,O-carboxymethyl chitosan). The PMSC CAPNs, which are different from typical hydrogels, will give responses to the changes of surrounding media by forming the double-phase isomerization. Based on the morphological reorganization, the specific properties including surface roughness, topography, free energy, polymer composition, electrostatic forces and the mechanical properties of the bulk will be generated and they all play large roles in the influences on the cell behaviors. Thus, in this study, based on the chemical, physical, and biological investigation between PMSC and normal human fibroblast, the cell-substrate interaction was identified for the optimization on the application in the wound bed preparation.
Automated Manufacturing of Tailored 3D Scaffolds to be Used for Tissue Engineering Applications

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Tissue engineering (TE) is an interdisciplinary field based on engineering and life science which is aimed at the development of biological substitutes that restore, maintain or improve tissue functions. One of the most exciting and promising approach in TE is the development of 3D scaffolds which serve as a substrate for cells to attach, proliferate and form new tissues or organs. In this regard, the development of a highly porous biodegradable 3D scaffold with a tailored architecture becomes essential for the understanding of how scaffold architecture affects the tissue regeneration. Unfortunately, current scaffold fabrication techniques use organic solvents (which is detrimental to cell survival) or are only used at research scale; thus, the scaffolds cannot be massproduced. In order to meet the prospective patient needs, in this work we present a new revolutionary automated and cost effective scaffold manufacturing process. With the development of this new technology it is possible to obtain completely interconnected FDA-approved scaffolds based on synthetic polyesters with pre-designed pattern, shape, porosity and pore size. As confirmed by scanning electron microscopy, mechanical tests and stem cell interaction, the scaffolds resulting from this automated manufacturing process are good candidates to be used as a three-dimensional substrates for the growth and proliferation of human mesenchymal stem cells for achieving a successful human cartilage replacement therapy.
Successful regeneration of annulus fibrosus (AF) tissue demands recapitulation of the anatomic hierarchy, composition and the most important mechanical functions. Several attempts have already been made using synthetic and other natural polymers; however failed to achieve the lamellar architecture of the native AF tissue. Thus simulating the structure-property relationship of the AF tissue could successfully engineer an artificial matrix with complete functional equivalence to the native components. Multilamellar criss-cross architecture silk fibroin fibrous scaffolds were designed using a winding machine and functionalized with chondroitin sulfate. The chemical crosslinking between silk and CS was characterized by ATR-FTIR, NMR and ninhydrin assay. The silk and silk-CS scaffolds were cultured with de-differentiated goat chondrocytes upto 6 weeks. Engineered constructs were analyzed at regular intervals after 1 week, 3 weeks and 6 weeks by MTT, SEM, TEM, H&E, IHC, biochemical estimation (GAG and Collagen), MALDI-TOF/TOF, qRT-PCR, compression and tensile testing. The lamellar scaffolds could successfully serve as a template for the alignment of cells and the newly synthesized ECM to deposit along the ridges and grooves of the silk fibers. Enhanced metabolic activity and ECM production was observed in silk-CS constructs, compared to only silk- constructs. Differential ECM analysis by MALDI-TOF/TOF showed the expression of cartilage specific proteins which was further validated by RT-PCR. Replicating the anatomic orientation of cells and ECM and surface decoration of silk scaffolds with CS resulted in increased mechanical properties, thus simulating the structure-function properties of the native AF tissue.
The Catalyst-Assisted Two-Stage Synthesis of Poly(glycerol-co-sebacate-co-ε-caprolactone) Elastomers as Potential Subacromial Adhesion Barriers in Rotator Cuff Repair

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There has been an unmet need for biodegradable adhesion barriers in many surgical procedures. In this study, Poly(glycerol-co-sebacate-co-ε-caprolactone) (PGSCL) elastomers were synthesized for the first time from respective monomers. The structural analysis of PGSCL elastomers by Nuclear Magnetic Resonance (\(^1\)H-NMR) and Fourier Transform Infrared Spectroscopy (FTIR) revealed that the elastomers have high number of hydrogen bonds and cross-links. X-ray diffraction (XRD) and thermal analysis indicated an amorphous state. Differential Scanning Calorimeter (DSC) analysis showed that the elastomers has a glass transition temperature (\(T_g\)) of -36.96 °C. The Young’s Modulus and compression strength values were calculated as 46.08 MPa and 3,192 MPa, respectively. Calculations based on acid number and end groups analysis revealed a number average molecular weight of 148.15 kDa. Even though the foaming studies conducted by using supercritical CO\(_2\) resulted in a porous structure; the obtained morphology tended to disappear after 48 hours, leaving small cracks on the surface. This phenomenon was interpreted as an indication of self-healing due to high number of hydrogen bonds. The PGSCL elastomers synthesised in this study are flexible, robust to compression forces, and has a self-healing capacity. Thanks to good biocompatibility and poor cell-adhesion properties, the elastomers may find diverse applications where a post-op adhesion barrier is required such as in rotator cuff repair surgery.
Naturally derived hydrogels are frequently employed for biomedical applications because of their properties similar to the extracellular matrix of the natural tissues. Alginates are probably the most extensively studied and characterized hydrogels for applications in tissue engineering and regenerative medicine fields. The gelation reaction of alginate is produced when cations such Ca$^{2+}$ diffuse into solution and interact with specific segments of the polymer chains. In the present work, polymeric fibers were produced by wet spinning. The method consists in immersing the needle of a disposable syringe loaded with the alginate aqueous solution, into a calcium chloride cross-linking solution. To obtain the optimum hydrogel formulation, several aspects were considered such as concentration of both alginate and calcium chloride. Different processing parameters were considered to determine the best conditions required to achieve the most adequate response in terms of mechanical stability of the produced systems. Morphology, size and shape of the produced fibers were observed by light microscopy and the mechanical properties were evaluated by tensile tests. In-vitro weight loss tests were carried out to evaluate the water content of the fibers. The release properties of the fibers were tested, using albumin as model molecule, in order to evaluate the use of the fibers for the production of bioactive scaffolds. Cell encapsulation experiments were performed to assess the viability of cells incorporated into the developed hydrogel fibers and to optimize the encapsulation conditions. Fiber meshes were prepared and cell culture tests were performed to investigate the ability of the produced systems to support cell adhesion and proliferation.
Photodegradable Multi-Functional Hydrogel for the Directed Growth of Neural Precursor Cells

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The design and development of artificial niches to recapitulate in vitro the spatial and temporal features of complex extracellular matrixes (ECM) are of great interest in order to modulate sophisticated cell behaviours which are often associated with a cell’s environment. Here, we introduced a novel multi-component hydrogel material that consists of heparin and polyethylene glycol peptide conjugate which combines matrix metalloproteinase and photosensitive modules. The light-directed degradation can modulate spatial characteristics of this hydrogel matrix which can be further shaped by cell secreted proteases, while the high concentration of heparin can provide various morphogens to direct cell fate decision. This novel bio-hybrid hydrogel was used to develop multilayer materials by formation on a glass substrate, which was previously covalently modified with cell attachment signal peptides. Micro lens array patterning was used to produce defined channels and wells by photocleavage of the hydrogel upon laser irradiation. Photodegradation of the gel exposed the signal peptide on the surface of the glass creating a strong biofunctional contrast between the bottom and the walls of the wells and channels; thus providing a compartment model for spatial features of ECM. Use of the proposed model to culture neural precursor cells has revealed how the cells fate can be directed by manipulating the cell compartment while keeping the other biological and physical characteristics of the system constant. The proposed approach describes a simple method for creating various matrix architectures with defined biochemical functions, which allow us to generate different niche-like environments to direct cell growth and differentiation.
Development of a Modified Suture Technique which Transmits Sufficient Load on a Tissue Engineered Cell Seeded Collagen Construct in situ to Affect Matrix Remodelling: An in vitro and ex vivo Study

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Tendon in its natural state attaches a muscle to bone and is always under intrinsic tension. When complete rupture takes place due to trauma then the ruptured end releases tension and contracts proximally and distally to create a gap. When a gap is too large to be sutured under tension tendon grafts are used to bridge this gap. We have developed a highly reproducible, rapid process technique to manufacture compressed cell seeded type I collagen constructs to replace tendon autografts. Previously we have tested these constructs in vivo in intercostal spaces of a lapine model to prove immunocompatibility but, material properties of the engineered constructs are currently unsuitable to withstand complete load bearing in vivo. A modified suture technique has been developed to withstand physiological loading and off load the artificial construct whilst integration occurs. This modified suture method allowed only partial load to be transferred onto the construct. Lapine tendons (n= 56) were used to test mechanical strength of repairs and a FEA stress model was built with COMSOL 3.5 software. The break point for modified suture technique with tissue engineered collagen construct in situ was significantly higher 50.62N compared to standard modified Kessler suture [12.49N (p<0.05)]. To test the effect of the partial load on tenocytes mechanobiology was studied under static and 10% cyclic loads using custom designed CFM and t-CFM with immunohistology and matrix remodeling gene expression as quantifiable outcomes. Mechanobiology studies showed that endogenous matrix tension was maintained and also showed significant upregulation of matrix remodeling genes COL1 (RQ 7.2), COL3 (RQ 0.8), Tenomodulin (RQ 2.3) and TGF-b (RQ 4.8) [p<0.05]. We are in the process of testing the cell-seeded tissue engineered tendon in a lapine model in the Achilles tendon using the modified suture technique developed for integration and function. We hypothesize that the 3D cell seeded construct will render a microenvironment for tenocytes and speed up neo-tendon formation and matrix synthesis in vivo.
Injectable Nanofibrous Polycaprolactone (PCL)/ Collagen Bone Augmentation Material for Irregular Shape Bone Defects

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The stabilization of irregular shape bone fractures demands for a new generation injectable biomaterials possessing proper biological, mechanical and structural properties to host cells and to regenerate bones at the injected area. The objective of this study is to develop a novel injectable biomaterial as bone filler containing nanofibrous PCL synthetic polymer and collagen Type-1 natural polymer along with mesenchymal stem cells (MSCs) and bone morphogenic protein-2 (BMP2), to create a three-dimensional environment for bone cell-ingrowth, proliferation and differentiation. PCL nanofibers interspersed collagen (PN-COL) scaffolds, manufactured with varying PCL concentrations of 0%, 1% and 6% (w/v), were evaluated with respect to their physicochemical, morphological and biological properties. Specifically, the scaffolds were characterized in terms of cell viability, proliferation and differentiation along with structural stability, chemical composition, internal architecture and protein retention within the composite scaffold. Cell cytotoxicity, compatibility, proliferation and osteogenic activity data proved that introduction of PCL nanofibers into the collagen matrix increases the osteoconductivity of the scaffold. The encapsulated protein within PN-COL scaffold demonstrated significantly slower release profiles over 21 days in comparison to collagen scaffold without PCL; and higher protein retention was obtained with increase in PCL concentration incorporated in the scaffold. In addition, BMP2-encapsulated PN-COL scaffolds are current being tested in vivo to investigate biocompatibility, osteoinductivity and vascularization of the scaffold at the defect site.
Gelatin/Albumin-Based Hydrogels Scaffolds By Two-Photon Polymerization

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Developing 3D hydrogel scaffolds capable of promoting cell viability and important cell-extracellular matrix (ECM) interactions is of great importance for Tissue Engineering (TE) applications. To mimic the biochemical and structural complexity of ECM, two-photon polymerization (2PP) shows great promise to fabricate ECM-biomimetic hydrogels because it allows the fabrication of complex user-dictated shapes with micrometer-scale resolution. Poly(ethylene glycol) diacrylates (PEGDA) have been widely used for 2PP fabrication of hydrogels due to their high reactivity. However, the residues of PEGDA and unreacted acrylate groups are potentially cytotoxic. For most TE applications, monomers with low cytotoxicity are in demand. Our previous work proved that vinyl esters (VEs) are much less cytotoxic than their (meth)acrylates references. Although VEs are generally not as reactive as acrylates, the thiol-vinyl ester photo-click reactions are robust enough for efficient 2PP applications. Since it is hard to assemble ECM-biomimetic hydrogels using synthetic monomers alone, proteins with thiol and VE functionalities were prepared for use in thiol-ene photo-click hydrogels. Presented are the synthesis of vinyl ester derivatives of gelatin hydrolysate, reductive disulfide cleavage of bovine serum albumin, and 2PP fabrication of gelatin/albumin based hydrogel scaffolds using novel water soluble 2PP initiators. The vinyl ester modified gelatin/albumin mixture could be photopatterned at high writing speed and low laser power providing high resolution microstructures. Acknowledgement. The support by the China Scholarship Council (CSC No. 2009688014) and the European Commission in the framework of Marie Curie IEF (project VINOBONA, No. 297895) is gratefully acknowledged.
In Vitro Cytotoxicity Assessment of Pyrolytic Carbon/Ti6Al4V Film

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Carbon/carbon (C/C) composites are considered as prospective biomaterials for orthopaedic use due to their stability in the physiological environment and excellent mechanical properties. In this work, pyrolytic carbon/Ti6Al4V film was applied on C/C composites to improve their biocompatibility. The film was prepared in two steps: (i) chemical vapour deposition was performed on C/C composite specimens using natural gas as the precursor with partial pressure of 30 kPa and N2 as the diluent. CVD was performed at 1373 K, 0.1-0.2 s residence time and 8-h deposition time; (ii) the C/C specimens with pyrolytic C were coated with Ti6Al4V film using magnetron sputtering technique. During the coating process, the chamber pressure of 3 × 10−2 mbar, sputtering power of 500 W and sputtering time of 5 h were used. From the scratch test, the bonding strength between the film and the composite reached 80 MPa. The pyrolytic C/Ti6Al4V film fully covered the surface of C/C composites with a spherical morphology, exhibiting a uniform and dense surface structure. The shear stress of the film is greater than the bonding strength between the C/C composite implants and bone after implantation. The cell response to the film was studied by analysing the morphology and proliferation of MG63 cells. Most of the cells showed slice morphology and attached to the film tightly by filopodia. The cell spreading and proliferation on the pyrolytic carbon/Ti6Al4V film was better than that on uncoated C/C composites. It can be concluded that the pyrolytic C/Ti6Al4V film prepared by combination of CVD and magnetron sputtering have a strong bonding strength to C/C composites. The pyrolytic C/Ti6Al4V film improves the surface structure and biocompatibility of C/C composites. Acknowledgment: this work was funded by FP7-PEOPLE-IRSES project ABREM.
Preparation and Characterization of a Novel Conjugated Hydrogel for Bioprinting Application

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Scaffold-free tissue engineering specially bioprinting method is emerging as an alternative to conventional technique using scaffold. This approach overcomes some major limitations of the previous methods such as random fabrication, lack of vasculatures, and so on. This method utilizes computer-controlled 3D printer to position the biological layers properly to construct structures. In this research, a novel hydrogel from alginate and fibrin were characterized for this application. Introduced hydrogel showed significant improvement in properties of both alginate and fibrin. Although alginate has good mechanical stability to preserve the 3D structures, the cell compatibility of this hydrogel, such as cell adhesion, proliferation and differentiation, is not acceptable. On the other hand, mechanical properties of fibrin is poor, however, it can be a suitable support for cells. Thus, it was proposed that the combination of Alginate and Fibrin hydrogel can be an appropriate support for cells in 3D structure used for bioprinting process. For this reason, we developed a novel protocol for conjugation of fibrin and alginate. Briefly, the solutions of these two hydrogels were mixed with three different portions (fibrin: alginate= 20:80, 50:50, 80:20) and conjugated with EDC. Fibrin-conjugated alginate with the portion of 50:50 and 80:20 did not have enough mechanical stability and were degraded. Therefore, the characterizations were conducted on FCA (20:80). Some analyses like swelling and degradation rate, Fourier transform infrared spectroscopy (FTIR), Scanning Electron Microscopy (SEM), DNA content, glucose concentration, gelation time in micro scale as well as mechanical properties in micro scale were conducted. The results confirmed that the novel hydrogel, Fibrin conjugated Alginate with the portion of 20:80, demonstrated the best physical, chemical and biological response to endothelial cells. As discussed above, this combination can be chosen as an appropriate hydrogel in bioprinting application.
Lotus-leaf-like Structured Poly (L-lactide-co-ε-caprolactone) for a Blood Compatible Surface
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The antithrombotic effects of super hydrophobic surfaces by lotus leaf structure and the various methods have been reported by many researchers. In spite of useful method for antithrombotic surface, thrombus formation remains a serious problem in the use of biodegradable polymers. In this study, biodegradable elastic poly (L-lactide-co-ε-caprolactone) (PLCL; 50:50) copolymer was synthesized and manufactured into films with lotus-leaf-like structures by a simple cosolvent casting method and template method. We evaluated whether lotus-leaf-like structured PLCL (L-PLCL) could be applied to artificial vascular grafts. Differences in the surface structures of the films, with respect to hydrophobicity and the lotus effect, and antithrombotic efficiency in platelet-rich plasma (PRP) were examined using scanning electron microscopy (SEM) and a contact angle meter. The contact angle with the lotus-leaf-like surface was approximately above 150°. Furthermore, the L-PLCL film yielded a lower platelet adhesion rate in PRP than that yielded by an untreated PLCL film. The L-PLCL surface manufactured by these simple methods could be applied to implantable medical devices and tissue engineering as a blood compatible treatment.
Biocompatibility and Antibacterial Properties of Selenium-doped Hydroxyapatite Coatings

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The hydroxyapatite (HA) coatings deposited on the surface of metal prosthesis and dental implants are widely used nowadays due to the good results observed in terms of improved osseointegration. This trend is expected to continue but with new requirements demanded for these HA coatings as an enhanced bioactivity and higher functionality with an added value. Thus, the properties of HA coatings can be varied by adding to their composition different elements. Selenium (Se) is an essential trace element whose compounds are considered to represent novel materials due to its anticancer and antibacterial properties. A pioneering study of the fabrication of selenium-doped carbonated hydroxyapatite coatings (iHA:Se) by using pulsed laser deposition (PLD) technique, is presented. Different proportions of Se were incorporated to obtain the iHA:Se coatings. Their physicochemical characterization, performed by SEM/EDS, FTIR, FT-Raman, Interferometric Profilometry and XPS, revealed the typical columnar growth of HA in globular aggregates and the efficient incorporation of Se into the HA coatings by the, most probably, substitution of SeO₃²⁻ groups in the CO₃²⁻ sites. Biological evaluation illustrated the absence of cytotoxicity of iHA:Se coatings with excellent proliferation of the MC3T3-E1 pre-osteoblasts, becoming higher than on HA coatings. At the same time microbiological studies with Staphylococcus aureus, Staphylococcus epidermidis and Pseudomonas aeruginosa were conducted to assess the effectiveness of the iHA:Se coatings at inhibiting the biofilm formation. The SEM evaluation and the counting of colony-forming units (CFUs) demonstrated these antibacterial properties.
Biodegradable Polymers Based on Chitosan and Gelatin as Substrata for Limbal Epithelial Stem Cells

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The aim of this work was to evaluate semi-synthetic biopolymers based on chitosan (CH) and gelatin (G) as potential in vitro carrier substrata for human limbal epithelial cells (hLECs). To that end, human corneal epithelial cells (HCE) were cultured onto different CH-G membranes. None of the polymers were cytotoxic and cell proliferation was higher when CH was crosslinked with G. Expression levels of corneal epithelial markers (K3, K12, E-cadherin, desmoplakin, and zonula occludens (ZO)-1) were better maintained in HCE cells grown on CH-G 20:80 membranes than other proportions. Consequently, CH-G 20:80 was chosen for the subsequent expansion of hLECs. Cells derived from limbal explants were successfully expanded on CH-G 20:80 membranes using a culture medium lacking components of non-human animal origin. The expression levels found for corneal (K3 and K12) and limbal epithelial stem cells (K15) specific markers were similar to or higher than those found in limbal cells grown onto the control substratum. Our results demonstrate that CH-G 20:80 membranes are suitable for the expansion and maintenance of stem cells derived from the limbal niche. These results strongly support the use of polymers as alternative substrata for the transplantation of cultivated limbal cells onto the ocular surface.
In situ Crosslinkable ELRs to form Devices with Embedded Cells

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In tissue engineering sophisticated stealthy materials as (ELRs) the Elastin-like recombinamers have been studied for their potential excellence; in fact the recombinant assembly of their modular molecules allows combining in their own sequences several attractive mechanical, biocompatible, bioactive, biodegradable properties in addition to their intrinsic stimuli-responsive nature. One of the drawbacks of most scaffolds used for tissue engineering is its surgical implantation, for many medical applications, injectable hydrogels would be preferred because could be restored any desired shape at the site of the injury and minimizing the invasiveness of the procedure and incorporate therapeutic agents and encapsulate cells, amongst others. This work describes the use of a two-component system for the formation of injectable hydrogels generated "in situ" whose applicability is focused specifically on tissue engineering and regenerative medicine We obtained hydrogels in physiological conditions by crosslinking based on copper catalyzed azide-alkyne Huisgen 1,3-dipolar cycloaddition. MSCs viability and proliferation was quantified by LIVE/DEAD and Alamar blue® over a period of two months. Mechanical tests were performed on rheometer. We have developed a system to obtain injectable ELRs hydrogels that allow the proliferation of stem embedded cells for at least 60 days. These hydrogels showed to be non-cytotoxic and highly biocompatible, they may encapsulate and culture stem cells and thus be employed as injectable implants for tissue regeneration procedures.
rGO/PEDOT/BFDMA Hybrid Composites on Neural Electrode for Neural Tissue Engineering

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Despite recent advances, there are major hurdles to overcome before cortical neural prosthetics can become a viable therapeutic strategy. The injury response to the insertion of these devices results in eventual loss of recorded neuronal signal, preceded by inflammatory responses at the injury site. Variations in probe electrode composition and designs are being investigated to enhance probe viability. In this study, strategies integrating disciplines of chemistry, biology and engineering will be employed to explore methods to advance the field of invasive cortical devices in neural tissue engineering. The first effort involved the development of completely new materials for modifying the surface of neural electrode. A novel porous structure rGO/PEDOT/BFDMA(Bis-(11-ferrocenylundecyl) Dimethylammonium Bromide) conductive functionalized hybrid composites will be developed and processed onto the neural electrode surface by a new “one step electro-deposition” method. Under controlling the ratio of rGO/PEDOT and variations of electro-deposition, the surface morphology will change in affecting bio-compatibility and detection limitations. The conductive rGO/PEDOT provides good cell-to-substrate interactions and high conductivity. Moreover, the ability of detecting neural signals is enhanced due the high surface area ratio by porous structure. The two ferrocenyl groups of BFDMA work as feelers help to detect more detail neural signals. These results demonstrate that the new composite material will be characterized as neural electrodes as potential application for tracking neurological disorders in tissue engineering.
Click Copper Free ELRs Hydrogel for Vascular Graft Device

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In the last two decades several techniques have been used to treat vascular diseases resulting in stenosis, such as stenting and vessel replacement. One of the greatest problems in the use of stents is the recurrence of luminal narrowing due to in-stent restenosis. Elastin-Like Recombinamers (ELRs) are tailored recombinant polymers, totally biocompatible, elastic, with a thermoresponsive behavior. Furthermore ELRs can be functionalized with specific sequences to achieve desired biological properties. In this work, new ELRs gels obtained by a copper-free click reaction are presented. Gel formation can be carried out under physiological conditions with a completely cell friendly process. These gels have been used to embed commercial non-resorbable stents in order to exclude the atherosclerotic plaque from the luminal side of the vessel and create an intact functional endothelial cell layer. Mechanical tests under physiological conditions revealed a high mechanical stability of the novel constructs. ELRs with different biofunctionalization have been tested in order to optimize the attachment of human umbilical vein endothelial cells (HUVECs) to obtain a complete endothelial lining in vitro prior implantation and, therefore, in vivo physiological haemocompatibility. The non-thrombogenicity of the endothelialized ELR-stents has been confirmed with blood tests under physiological flow conditions. Future developments include the use of ELRs with specific sequences to capture circulating endothelial progenitor cells in order to obtain the endothelial lining in vivo. In this way off-the shelf stents can be produced.
Citric Acid-Cross-linked Hydrogels: Potentially Self-Mineralizing Scaffolds for Bone Tissue Engineering

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Elastin-like recombinamers are versatile polypeptides known to be biodegradable, biocompatible, and bioactive when specific peptides such as RGD for cell adhesion are introduced in their sequence. Although these polymers have been proven to self-aggregate at low temperature, covalent cross-linking is needed to guarantee material stability and easy handling. In the present work, citric acid was chosen as a bridging agent since most of this compound (up to 80%) in humans is found in bone, our target tissue. Recent evidence has suggested that this local accumulation is due to its in vivo implication in stabilizing hydroxyapatite crystals to mechanically optimal size. Furthermore, citrate has been shown to be able to induce calcium phosphate nucleation in vitro, making it an interesting molecule to be included in scaffolds for bone tissue engineering. Water-soluble carbodiimide chemistry was used to make citric acid reactive towards lysine residues in the polymer sequence and cross-linking by means of peptide bonds was achieved at 37°C. Hydrogels displaying differences in polymer aggregation (globular, fiber-like or almost continuous matrix structures) and elastic modulus, two attributes known to affect cell behavior, were obtained by changing reaction conditions. After alternate soaking protocol, citric acid-containing gels showed massive calcium phosphate (with Ca/P close to that in bone) deposition when compared to glutaraldehyde-cross-linked gels. These results show that (i) hydrogels with different cell-instructive features can be achieved and (ii) using citric acid as cross-linking molecule can add mineralization capacity to otherwise poorly mineralizing polymers.
Thin Calcium Phosphate Coatings Enhanced Bone Response to Titanium Implants in Osteoporotic Conditions

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The bone response to titanium implants is significantly affected by medical factors, e.g. by postmenopausal osteoporosis. A more in-depth understanding of the biological activity of osteoporotic bone around implants will have considerable industrial and scientific importance. Consequently, titanium implants with or without a radiofrequency magnetron-sputtered calcium phosphate (CaP) coating were installed in the femoral condyle of ovariectomized and sham-operated rats. At 8 weeks post-implantation, mechanical testing and histomorphometrical as well as micro-CT evaluation were performed. Fluorochrome labeling at 3, 5, and 7 weeks post-implantation were used to study the dynamics of bone response at the implant interface. Micro-CT bone volume (%BV) and histomorphometrical bone area (%BA) were lower around control implants in osteoporotic rats (BV= 38.9%, BA= 35.4%) compared to sham-operated rats (BV= 65.3%, BA= 47.7%). Interestingly, CaP-containing surface layers enhanced implant fixation (push-out testing) compared to non-coated implants in osteoporosis (58.8 N vs. 19.9 N, respectively) as well as in sham-operated (108.0 N vs. 70.6 N) groups. Bone-to-implant contact (%BIC) was higher for CaP-coated implants compared to non-coated controls in osteoporosis (61.3% vs. 46.7%) and in sham-operated (86.9% vs. 64.1%) groups. For bone activity dynamics, CaP-coated implants showed a significantly increased bone activity in close vicinity to the implant surface at 3 weeks of implantation. The study confirmed that a thin calcium phosphate coating is an effective compensatory approach to improve the bone-to-implant response in both osteoporotic and healthy conditions.
Devitalization by Apoptosis-induction for the Generation of Extracellular Matrices with Well-preserved Instructive Properties

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Devitalized engineered extracellular matrices (ECM) are used in a variety of regenerative medicine applications. Existing devitalization strategies rely on cell lysis and generally result in a variable but significant impairment of the ECM structure/composition. As an alternative, we aimed at activating the apoptotic pathway in order to devitalize engineered matrices while preserving its properties. Towards this goal, we generated an immortalized human Mesenchymal Stromal Cell (hMSC) line further transduced with an inducible caspase 9 apoptotic gene. The cell line was shown to retain primary hMSC properties (phenotype, differentiation and immunomodulation capacity) while being inducible toward apoptosis (killing efficiency >95%). Cells were seeded on ceramic scaffold and were shown to proliferate, differentiate and to deposit a dense ECM during 3D perfusion bioreactor culture. The apoptosis induction allowed for efficient devitalization while preserving the secreted matrix, especially when compared to a Standard Freeze & Thaw technique. The cell-free ECM coated constructs were implanted in an animal cranial defect model where such “apoptized” constructs induced bone regeneration. The use of apoptosis appears as an innovative and relevant method to devitalize engineered ECM. In our set-up, the use of an immortal but death-inducible hMSC line allows to generate cell-free osteogenic graft in a streamlined process, directly addressing standardization issues typically raised by the use of primary hMSC. More generally, the validation of the apoptotic approach paves the road for the study of specific ECM properties, by preserving its integrity while depleting the cellular fraction.
Adipose Tissue Promotes Collagen Production by Fibroblasts by Secretion of PGF$_{2\alpha}$

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Adipose tissue has an endocrine function, next to energy storage and isolation, and contains macrophages, which can be divided into pro-inflammatory macrophages (M1) or anti-inflammatory/repair macrophages (M2). We investigated how adipose tissue influences tissue repair focusing on collagen production by fibroblasts. Adipose tissue of 13 different donors was cultured (50 mg/ml) for 24 hours to make fat conditioned medium (FCM). Fibroblasts (50,000 cells/cm$^2$) were cultured in FCM for 4 days to analyse gene expression of Plod2 (encoding LH2b, an enzyme involved in collagen cross-linking), and α smooth muscle actin (Asma), and 7 days for collagen production. 10 µM of SB505124 (TGFβ receptor/ALK5 inhibitor) or 1 µM AL8810 (PGF2α receptor inhibitor) was used to study the involvement of TGFβ or PGF2α. Adipose tissue used to make FCM contained many CD68 and CD206 positive cells but less CD86 positive cells.

FCM increased collagen production 2 times (p<0.05) and Plod2 6 times (p<0.01) in fibroblasts while Asma was decreased (p<0.01). Inhibition of canonical TGFβ signalling did not change the effect on fibroblasts. Blocking PGF2α however, normalised collagen production and Plod2. Adipose tissue used to make FCM contained many CD68 and CD206 positive cells but less CD86 positive cells.

In conclusion, adipose tissue can play a role in tissue repair by secretion of factors that promote production of collagen by fibroblasts, that is probably also more cross-linked, without differentiating them to myofibroblasts (based on Asma). PGF2α is partly responsible and repair macrophages (M2) might contribute to these effects.
Towards New Extracellular Matrices for Biocardiac Tissue Engineering

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Due to the permanent lack of organ donors the regeneration of tissues or the replacement of failing or malfunctioning organs is an essential and upcoming challenge. One approach to build up organ implants is the colonisation of scaffold materials with bioactive molecules and adequate cells. Polymers of natural origin and among them carbohydrates are very promising as scaffold materials. They are fully characterised, own intrinsic biocompatibility, and provide chemical versatility. Furthermore they are - in contrast to the commonly used scaffold material Matrigel™ - not from animal sources and therefore not potentially xenogenic. In our recent studies we investigate hydrogels from spontaneously in situ hardened biopolymers allowing an simultaneous and homogeneous encapsulation of cells. Alginate and hyaluronic acid were first transformed into aldehyde and hydrazide derivatives. Mixing both components without any additives at rt in a one to one ratio generated colourless, water stable gels in a matter of minutes. The in situ hydrogelation could be performed in water, isotonic sodium chloride or various culture media. Depending on the degree of derivatization and the utilized media the gelation time could be modified. In order to mimic the extracellular matrix of organisms the decoration with bioactive molecules is necessary. Therefore, a metal-free cycloaddition “click” reaction was applied to bind RGD-peptides. Combining both chemical strategies, the in situ gelation process and decoration with bioactive molecules generates a complex artificial extracellular matrix from natural occurring polymers.
**Albumin-Based Scaffold Promote the Proliferation and Migration of Human Dermal Fibroblasts**


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Loss of integrity of large portions of the skin due to injury or illness may result in considerable disability or mortality. The goal of this study was to fabricate a novel sponge-form proteinous scaffold from egg white albumin and investigate the behaviour of fibroblasts within the scaffolds.

Egg white powder dissolved in acetic acid (4 wt%) and samples were placed in -80°C freezer overnight and subsequently lyophilized for 24 h. The sponges were cross linked chemically for 24 h in EDC/NHS solution. Morphology and pore size distribution were characterized with Scanning Electron Microscopy (SEM). Fourier-transformed infrared (FTIR) and Differential Scanning Calorimetry (DSC) data obtained to determine the extent of crosslinking. Water uptake and degradation behaviour of the samples studied over 24 h and 21 days, respectively. To evaluate the cytotoxicity of the samples and proliferation of fibroblasts, the MTS assay was performed in 3, 7 and 14 days following cell seeding. Morphology, proliferation rate and migration of the cells in scaffolds were assessed in day 14 with H&E staining, immunohistochemistry (Ki67 antibody) and propidium iodide (PI) staining. SEM images demonstrated interconnected pores with mean diameters of 30-50 μm and over 90% porosity. FTIR and DSC assessments revealed formation of new crosslink bands and alteration in shrinkage temperature of samples after crosslinking. Cross linked scaffolds had a water content and degradation of about 90% and 70%, respectively. EDC-cross linked scaffolds demonstrated no significant toxicity. The proliferation and percentage of Ki67+ cells enhanced during 14 days post seeding. Fibroblasts migrated approximately 1 mm through the depth of scaffolds.

This porous scaffold can enhance the proliferation and migration of fibroblasts and can be a good candidate for skin tissue engineering applications.
Modulating Bioactivity of pH Dependent Polyelectrolyte Multilayers Prepared from Natural & Semi-Synthetic Glycosaminoglycans

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Here, layer-by-layer method, based on alternating adsorption of oppositely charged polyelectrolytes was used for creating biomimetic thin films by incorporation of biogenic molecules on glass as model biomaterial. Polyelectrolyte multilayers (PEM) of heparin as well as synthetically made cellulose sulfates (Cs) as polyanions and chitosan as polycation were prepared at pH 4 and 9. Cellulose sulfates of varying sulfate content were applied as they are highly bioactive like heparin showing mitogenic and osteogenic activity. pH variation was applied to control the physicochemical and biological properties of PEM. The resulting PEM were characterized by water contact angle measurements (WCA), quartz crystal microbalance (QCM) and surface plasmon resonance (SPR) measurements. Cellular investigations were done using C2C12 cell line.

WCA results showed distinct pH-dependent changes in wettability of multilayer by adsorption of different polyelectrolytes, attributed to their functional groups. SPR and QCM also showed layer built-up by material adsorption also dependent on pH value and type of adsorbed polyanion. Cell experiments demonstrated that multilayers assembled at pH 9 were more adhesive for C2C12 cells than pH 4 layers and this might be due to involvement of adhesion proteins like fibronectin which were also well adsorbed on pH 9 layers. To conclude, bioactive multilayers were successfully prepared from semi-synthetic glycosaminoglycans like heparin at specific pH conditions. The effect of sulfation degree of polyanions was seen in physical properties of PEM and also on C2C12 cells.

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Characterizing the Viscoelasticity of Tracheal Rings under Multiple Loading Configurations

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The trachea, a cartilaginous tube that regulates airflow to the lungs, is occasionally treated as a linear elastic material to facilitate some applications. However, recent studies have noted the importance of accounting for its viscoelastic properties when investigating tracheal trauma or engineering replacements. Because tracheal damage can occur as a result of compression (blunt trauma) or tension (increased internal pressure from a mechanical ventilation aid), it is desired to assess the viscoelastic properties of trachea cartilage under various loading conditions. Here, a mechanical testing system (Bose Corporation) was used to load rings of tracheal cartilage under compression and tension. To assess the creep response, rings were compressed either axially or longitudinally with 120 g of force and held while the nonlinear strain response was allowed to equalize. The compressive strength in the longitudinal direction was found to be greater than in the axial direction. Stress relaxation tests were carried out on samples under axial tension or compression. Samples were pulled or compressed to 25 % strain and held while the nonlinear stress response was allowed to equalize. The tensile strength of the tracheal ring was found to be greater than the compressive strength. Finally, samples under axial compression were cyclically preconditioned with 25 g of force at 1.0 Hz for 10 cycles. The samples were then compressed to 50 g of force (corresponding 30% strain) and the load was subsequently removed. Sample hysteresis was observed as a lag between the loading and unloading curves. Overall, this study illustrates the importance of trachea viscoelasticity as evidenced by pronounced creep, stress relaxation, and hysteresis.
A Fibrin/Alginate Smart Matrix Scaffold for Full Thickness Skin Reconstruction

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The concept of artificial skin for reconstruction of full thickness skin-loss wounds is clinically appealing. The term suggests an effective, convenient, off-the-shelf biomaterial to instantly replace lost skin. A substantial number of techniques and clinical products developed for skin reconstruction over the past twenty years can be grouped into several strategies. However, limitations of these products, and underlying strategies, means the clinical imperative remains. Smart Matrix™ (SM) is a new fibrin-based dermal scaffold, which integrates and vascularises rapidly, and subsequently enables ‘regenerative-type’ tissue remodeling. Significant pre-clinical experience in the porcine model now allows SM appraisal in relation to current products. The fibre mesh structure of the SM biomaterial and porosity support rapid vascularisation and cellularisation (>2x Integra) in a full thickness wound model. Importantly, this involves a vasculogenic mechanism and occurs throughout the scaffold depth (1-2mm). This process enables one step full thickness reconstruction using a split-thickness over-graft onto the scaffold. In vitro studies suggest a role for cytokine and growth factor signaling, and fibroblast/myofibroblast differentiation data indicate ‘regenerative-type’ characteristics of tissue remodeling, corroborating histological outcomes. Collectively, these results demonstrate that a biomaterial scaffold can selectively regulate wound healing and tissue reconstruction in a regenerative trajectory.
Enhanced in vitro Biological Response of a New Porous Alumina-Zirconia Composite for Bone Tissue Engineering

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Ceramic materials such as zirconia and alumina are used widely for bone repair due to their exceptional suitability for load-bearing applications. However, limitations such as poor bioactivity, have led to the development of advanced alumina-zirconia composites which may display improved biological features. The aim of this study was to develop a new sintered alumina-zirconia composite and to investigate its biological response with the MC3T3-E1 pre-osteoblasts.

The alumina-zirconia composite was produced by sintering powders. We determined porosity by the Archimedes method, pore size distribution by SEM and mechanical properties were characterized by compression tests. Cell proliferation was assessed by the PrestoBlue™ assay and fluorescence microscopy. Cell adhesion and morphology were observed by SEM. Collagen levels and alkaline phosphatase (ALP) activity were measured to assess extracellular matrix and osteoblast differentiation.

The composite was a mixture of tetragonal and monoclinic phases with 7.5 GPa Young modulus, 60% porosity and bimodal pore size distribution. Pre-osteoblasts adhered well, spread and proliferate on the composite. After 10 days, the entire sample’s surface, including pores, was covered with cells having numerous filopodia extensions attached to the surface and to each other. Abundant extracellular matrix formation was verified by collagen levels, whereas ALP activity was detected after 7 days.

The excellent cellular responses regarding adhesion, proliferation and differentiation on this new alumina-zirconia composite demonstrate its potential in promoting biological fixation and facilitating bone ingrowth, which is important in the clinical setting.
Currently, it is common to subject natural, protein-based scaffolds such as collagen to cytotoxic cross-linking procedures to improve their mechanical properties. However, this prevents the initial incorporation of cells which must then be seeded later. Here we describe two separate methods that utilise polymeric collagen (PC), a naturally cross-linked collagen fibril mesh. This is a purified suspension of pre-crosslinked fibrils as they occur in tissues. The first approach is reliant on a pH dependent aggregation of PC at pH 6-7 and can generate fibril alignment by applying sheering forces to the PC suspension during neutralisation. The alignment of collagen fibrils is particularly useful for the production of highly anisotropic constructs, such as tendon equivalents, but was found not to allow cell incorporation. However, a second approach based on a cold hydrogel formed from blending PC and monomeric, acid-soluble collagen, was developed as a cellular ready-cross-linked material. Sheer aggregation of collagen fibrils from PC:Monomeric collagen hydrogels (ratios 1:4, 2:3, 1:1 and 3:2) resulted in low yields of aligned collagen, but plastic compression (Brown et al, 2005) produced dense/tough collagen constructs with randomly aligned cross-linked fibrils. Human dermal fibroblasts within such compressed cold gels (ratios 1:4, 2:3 and 1:1) were incubated for up to 14 days demonstrating good cell viability.
Insulin-coated PCL/PLGA for Cartilage Regeneration

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Traumatic and non-traumatic cartilage damages are the common disabling human diseases worldwide. Absence of vascularization and presence of highly specialized matrix in hyaline cartilage usually inhibit proper cartilage self renewal and perfect regeneration. Tissue engineered scaffolds seeded or unseeded by cartilage-forming cells have been introduced as promising adjunct in cartilage repair. Various types of scaffold harboring different fibers, cells, and growth factor have been proposed for cartilage repair. In this study we have produced 4 types of scaffold for evaluation of degree of cartilage differentiation. Human nasal chondrocytes were seeded on PCL (polycaprolactone)/PLGA(poly-L-glycolic acid), PCL/PLGA coated with collagen, PCL/PLGA with Insulin, and PCL/PLGA coated with insulin and collagen. Collagen coating was approved by infrared spectroscopy (FTIR). Radioimmunoassay for Insulin showed continuous release in culture media until day 16th and afterward showed decrease. Scanning electron microscopy showed penetration and deep distribution of round cells within the scaffolds. Hematoxylin and eosin and alcian blue staining revealed chondrocyte-like cells on the scaffolds. The results of real time PCR for mRNA of collagen II and aggrecan showed higher effect of controlled-release of insulin on redifferentiation of chondrocytes. Immuno-fluorescent staining of scaffolds at day 21 showed that insulin containing scaffold had better and stronger expression of collagen and aggrecan in the cells located on the scaffolds. PCL/PLGA scaffold with controlled release of insulin showed the best condition as a three-dimensional scaffold for enhanced differentiation of chondrocytes. Despite increase in cell attachment in collagen-coated scaffolds, these scaffolds were not superior to insulin coated scaffold.
Many extracellular proteins have a fibrous structure with fiber diameters varying from nanometer to submicrometer scales. Electrospinning, which is a fiber fabrication technique by using electrical forces to produce polymeric fibers with diameters ranging from 2 nm to several micrometers, can be used to prepare materials mimicking the fibrous extracellular matrix proteins for potential use as tissue engineering scaffolds. In this study, Chitosan (CH) and its blends with poly(lactic-glycolic acid) (PLGA) and their combination with Hydroxyapatite (HAp) were used in the preparation of nano fiber structures. CH, CH-PLGA, CH-PLGA-HAp and PLGA nanofibers having fiber diameters of 180-525 nm were prepared. Resultant fiber mats were characterized by morphological, chemical, thermal, degradation and in vitro tests. Addition of PLGA increased the degradation rate of chitosan and 50% of CH-PLGA fibers was degraded in 4 weeks. Cell culture tests showed that all electrospun fibers promoted SaOs-2 cell attachment and proliferation. However, cell proliferation on CH-PLGA-HAp fibers was much higher compared to other fibers after 7 days demonstrating higher biocompatibility of the composite fibers in respect of stability and cell affinity properties.
Polycaprolactone/Poly(anthranilic acid) Scaffolds for Improved Cell Adhesion in Tissue Engineering Applications

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Mimicking extracellular matrix (ECM) with the electrospun scaffolds are important method in tissue engineering applications. Polycaprolactone (PCL) is a biocompatible polymer that can easily be electrospun and it is preferred in many tissue engineering applications due to its elasticity, and mechanical properties. However, it has no functional group that reduces the cell attachment to the surface of the electrospun scaffolds. In this study, firstly PCL scaffolds were electrospun with chloroform and dimethylsulfoxide solution with the weight ratio of 15% (w/w). After their characterization with spectroscopic techniques, microscopic observations and H-NMR results, PANA/PCL scaffolds were processed by electrospinning technique successfully. Furthermore, the nanofibers will be used in cell attachment experiments. Before that, bovine serum albumin will be immobilized on the surface of the scaffolds using EDC/NHS coupling mechanism to obtain protein rich surfaces for future cell attachment purposes.

Figure 1: The electrospun scaffolds; PCL scaffolds (a) PANA/PCL scaffolds (b).
Physical Characterization of Micropatterned Collagen Films

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Scaffold production is important in terms of providing required architecture, bioactivity, mechanical strength and biocompatibility for tissue growth. Chemistry and topography of scaffold influence ECM secretion, and cell adhesion and the surface topography guides cells. Besides, physical cues affect cell proliferation and mechanical strength that are vital to obtain implant surfaces with tailored properties. In this study, collagen films with micro pillars and grooves were prepared by solvent casting on PDMS molds. Patterns on the collagen films were examined with SEM and degradation tests in PBS were performed to compare different crosslinking methods. Degradation tests showed that crosslinking with carbodiimide (EDC) and N-hydroxysulfosuccinimide (NHS) leads to low degradation rates (50% in 2 weeks) while crosslinking by UV radiation or use genipin were not as effective.
One of the most important fields of nanofibers use is biomedical applications. Our research group deals with a development of the ordered or random three-dimensional structured scaffolds with defined inner morphology of nanofibers. Fibers diameter can influence the porosity of the scaffold and thereby a cell adhesion, proliferation, migration, and differentiation. Nanofibers were fabricated by electroblowing method which combines electrostatic nanofiber production (“electrospinning”) with airflow around the spinneret. The vicinity of the emitter, optimal climatic conditions favorably influencing the spinning process are formed. Moreover, the speed and temperature of the air flowing around the nozzle affects the diameters of nanofibers. Nanofiber materials were fabricated from materials PVA, PEO, PCL, HA/PEO and HA. The effects of increasing air velocity in the vicinity of the spinneret reduced fiber diameter down to 50 %, whereas the increased air flow temperature reduced the viscosity of the solution spun and thus contributes to nanofibers production. Using electroblowing method allows you to change the morphology of nanofiber. The method can successfully spin solutions without the use of surfactants or other solvent systems, which are generally toxic and unsuitable/prohibited for medical applications. This is the only way in which hyaluronic acid can be spun in its native form.

**Keywords**
Electroblowing, Nanofibers, Scaffold, Biomedical Applications
Cells are intrinsically sensitive to local mesoscale, microscale, and nanoscale patterns of chemistry and morphology. In the body, the nanoscale structure of the extracellular matrix (ECM) provides a natural web of intricate nanofibers to support cells and present an instructive background to guide their behavior\(^1\). We use electrospinning to spin three-dimensional scaffolds which have nanofiber pattern in morphology and collagen/hydroxylapatite component in chemistry for the purpose of bone cell culture and regenerative tissue. Human-like collagen which is water-soluble, low-immunogenicity and good biocompatibility was used to electrospin. Dynamic deposition method was used to obtain hydroxylapatite coatings on the surface of collagen nanofibers. Deposit products are uniform distributed on each nanofiber inside of the scaffold. A collector covered with needles was used to collect nanofibers. Sample with 5mm thickness was spinned on needle-collector compared with 0.5mm thickness on a plane-collector in 2 hours.

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Artificial Graft for Eardrum Regeneration

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Eardrum perforation is a common otological problem. It can occur as a result of otitis media or trauma, leading to conductive hearing loss. The treatment for eardrum perforation aims to heal the eardrum tissue, restoring hearing and preventing possible infections. To date, the only available approach to repair eardrum defects are autologous implants, typically using temporal fascia as graft. However, the implanted tissue has short lifetime, impairing complete eardrum regeneration and showing low rate of success. The aim of this investigation is to create a new artificial graft for eardrum regeneration, consisting of an electrospun polymeric membrane cultured with human keratinocytes, thus allowing cell migration and tissue regeneration. The expected advantage is the proper tuning of implant lifetime, allowing eardrum regeneration and hearing repair. The proposed membrane consists of a blend of poly(L-lactic acid) (PLLA) and poly(D,L lactide-co-glycolide) (PDLG) cultured with a keratinocyte primary cells isolated from human foreskin. The methodology for keratinocyte culture consisted of 24 hours of cell proliferation followed by 12 days of cell stratification in a medium/air interface. The results showed that the membrane provided a successful environment for cell growth and epidermal tissue formation, facilitating cell adhesion, proliferation and stratification in different layers. The membrane also provided empty spaces for the deposition of elements secreted by the epithelial cells resembling a basement membrane. The proposed artificial graft could be an alternative therapy to treat eardrum perforations that are unable to heal naturally and dispensing from autologous donation.
Alterations in Electrospun Scaffold Topography provide Distinct Three-Dimensional Growth Conditions for the Main Structural Cell Types of the Airway Bronchiole

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Current airway in vitro models generally focus on the function of individual structural cells cultured in a 2D monolayer, or interactions between structural and immune cells in simple co-culture systems. There are limited examples of 3D models of the bronchial mucosa. This project aims to provide a new tissue engineered model of an airway containing the three main structural cells of the bronchiole (epithelial, fibroblast and smooth muscle) cultured on synthetic scaffolds optimized to mimic the natural extracellular matrix that the cells encounter in situ. Scaffolds are electrospun using the non-degradable polymer polyethylene tetraphthalalte (PET). Fibre diameter and orientation have been manipulated through alterations in the polymer concentration or method of polymer deposition creating fibres from nanometer to micrometer in diameter which can be deposited in either a random or aligned fashion. The nanofibre scaffold closely resembles the basement membrane found in the apical mucosal layer of the bronchiole on which epithelial cells grow. The microfibre scaffold mimics the porous sub-mucosal layer where fibroblasts are present, and the aligned fibers provide topological cues to produce a fully aligned sheet of smooth muscle cells found in the outer muscularis layer. Current data shows the individual scaffolds support the growth and differentiation of the different cell types within a 3D environment. Future studies will bring the individual scaffolds together to form a construct of the bronchiole held within a perfusion bioreactor to allow effective nutrient exchange and metabolic waste removal and providing a platform to investigate the interrelationships between the different cell types when cultured together.
Computational Design of Tissue Scaffolds via the Level-Set Method

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One of the key requirements of scaffolds is the balance between mechanical function and mass transport to aid biological delivery and tissue regeneration. Computational topology design and Solid Free-form Fabrication efforts made it possible to create scaffolds with controlled architecture. The Level-set based approach unlike existing standard topology optimization methods can overcome issues such as high computational demand and local minima problems resulting in a more efficient and generalized synthesis effort and hence better performing tissue-engineering scaffolds. Here we develop such a computational design tool based on the level set method for optimizing scaffolds topologically based on desired multifunctionality including elasticity, diffusivity, and permeability.

FEM model of the studied tissue scaffold is prepared in COMSOL Multiphysics. Response of the scaffold is analyzed using solid mechanics, general form PDE and fluid flow modules. These modules are integrated to solve related physical governing equations simultaneously. Level-set method is performed by utilizing Hamilton-Jacobi equation in the general form PDE module. A level-set surface is initialized and modified according to the sensitivity of the desired metrics with respect to material parameters in respective constituent modules. The change in the level-set surface is automatically reflected on the scaffold structure and an optimum structure with desired stiffness, porosity and diffusivity is obtained by finding an optimum level-set surface.

Results show that an optimum structure has been reached. Despite convergence challenges, by adjusting the artificial damping term and the time step, well-defined scaffold topologies for desired strength and release performance resulted in microstructural porous layouts with interconnectivity.

The numerical model shows that level-set method is an effective tool to improve the topology of tissue scaffolds. Fabrication of the designed scaffold and its in-vivo testing is currently underway.
Assessment of Silk and Silk/Nano-CaP Bilayered Scaffolds for Osteochondral Tissue Engineering

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Osteochondral defect (OCD) induced by trauma or disease (such as osteoarthritis) is one of the major problems in surgery. The ideal regeneration outcomes of OCD are not achieved yet by the traditional approaches. Tissue engineering is a promising strategy for this hinder. A bilayered scaffold architecture has been considered as an appropriate route for simultaneous regeneration of cartilage and subchondral bone in OCD. Previously, we developed novel silk and silk/nano-CaP scaffolds aiming for OCD regeneration. In the current study, the mechanical and degradation properties of these scaffolds were examined. The cytotoxicity was evaluated by culturing rabbit bone marrow stromal cells (RBMSCs) in the scaffolds for 7 days. The attachment and proliferation behavior of the RBMSCs on the bilayered scaffolds were evaluated in both basal and osteogenic culture media. The alkaline phosphatase (ALP) level of RBMSCs on each layer was tested. The result showed that the mechanical property of the bilayered scaffolds was comparable to the ones of single layered silk or silk/Nano-CaP scaffolds. The increased MTS results indicated that these scaffolds were not cytotoxic. The scanning electron microscopy (SEM) and live/dead staining images exhibited that RBMSCs adhered well on the surface of the scaffolds. The cells proliferated in the scaffold in both culture conditions. The RBMSCs in the silk/nano-CaP secreted higher ALP content as compared with the silk layer, in both basal and osteogenic condition. These \textit{in vitro} studies demonstrated that the silk and silk/nano-CaP bilayered scaffolds are good candidates for osteochondral regeneration.
Histogel: A New Biomaterial from Human Origin that Mimics Extracellular Matrix Properties

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In the field of tissue engineering, the repair of tissue defects needs an appropriate scaffold that provides a favorable microenvironment for the regeneration process. Sulfated GAGs (sGAGs) and hyaluronic acid (HA) are important extracellular matrix (ECM) components. In this sense, the extracellular matrix of Wharton jelly (WJ) present in umbilical cord is very rich in GAGs. Each of these components has special properties that could positively influence on the regeneration of several tissues. This work is focused in to define a new biomaterial for the generation of scaffolds based on WJ-derived HA and sGAGs, and ii, to determine the potential of the new biomaterial to develop a microenvironment able to direct the growth and differentiation of stem/progenitor cell populations for tissue regeneration. In this study, we report the process of extraction, separation and purification of GAGs from human WJ, the characterization of these separated GAGs (sGAGs and HA), the specific combination of different proportions of them in order to design natural 3D matrix hydrogel-type biomaterials for specific biomedical applications and, finally, the influence of these scaffolds on cell viability, differentiation and expression of various markers in some cell lineages. Our WJ-derived 3D matrix hydrogel-type biomaterial induces cell proliferation and increases the expression of collagen type II in adipose stem cells (ASCs), indicating chondral differentiation. In addition, an increase in the expression of collagen type I, fibrillin and fibronectin was found when the 3D matrix was used as scaffold for dermal repair. Furthermore, the new designed hydrogel increases the migration of both ASCs and fibroblasts when compared to a mimetic hydrogel composed by commercial HA and chondroitin sulfate (CS).
Recently bone tissue engineering studies have focused on the development of 3D scaffolds that can organize the tissue regeneration in natural way with appropriate porosity and reinforced the structure. Chitosan and its derivatives are widely used biomaterials for bone tissue engineering applications due to their porous structure, gel forming properties, ease of chemical modifications, biodegradable, biocompatible structure, antibacterial properties and high affinity to in vivo macromolecules. Furthermore, it has structural similarity to glycosaminoglycans which are the major components of the extracellular matrix. Silica particles can improve mechanical properties of polymers by providing enhancement in the structure, modulus and strength. Recent studies show that, silica content supports bone cell adhesion and bone tissue formation. In this study novel chitosan-silica composites were fabricated using natural and synthetic silica by freeze-drying and electrospinning. Composites were characterized by using SEM, mechanical test, swelling studies, protein adsorption assay and Indirect MTT assay in order to determine surface, morphological, mechanical properties and biological activity. Results showed that increasing silica content increased Young's Modulus of the composites (19.9-22.9 MPa) when compared to pure chitosan (13.5 MPa). Average pore size was obtained between 280-360 µm range. Protein adsorption increased with increasing silica content in 24h and 48h periods. Cell viability increased from 83% to 97% with increasing silica content when compared to negative control. In conclusion, three dimensional porous structures were obtained which shows some promises for bone regeneration applications.
Electrospinning and Characterization of Oriented PLA/PCL (70:30)/ Hyaluronic acid/Collagen Microfibrous Scaffolds for Tissue Engineering Applications

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Tissue engineering is an interdisciplinary field that aims to develop new biological substitutes to repair tissue injuries and restore their structure and function. This approach involves cell propagation in vitro and a subsequent cell seeding on 3D biodegradable scaffolds where the cells attach, grow and form the new tissue substitute as they differentiate. It is well known that the cell carriers, with their chemical, biological, architectural and physical properties, have a substantial effect on the behavior of anchorage dependent mesenchymal stem cells. Cells that interact with the fibrous extracellular matrix (ECM) have a semi-oriented morphology. Tendons, nerves and muscle fibers are oriented parallel to each other and scaffolds that mimic the natural ECM on the same scale can be produced and used to control cellular orientation.

In this study, oriented microfibrous scaffolds were produced by electrospinning for soft tissue engineering applications. Hyaluronic acid and collagen, the ECM components of most of the human tissues, were isolated from umbilical cord of human volunteers and were used in the production of the fibrous scaffolds. Semi-synthetic fibers were produced by adding a synthetic copolymer, PLA/PCL (70:30), to the biopolymer solution. The mechanical, thermal, and morphological characteristics of the fibrous mats were studied with a tensile tester in liquid medium at 37°C, differential scanning calorimetry and scanning electron microscopy, respectively. Mats with unidirectional fiber orientation were obtained; fiber diameter ranged between 0.5 and 1 micrometer. The Young’s moduli of the mats were determined as 1.313 ± 0.316 MPa for the synthetic mats and 0.886 ± 0.109 MPa for the semi-synthetic mats. Histochemical staining of the semi-synthetic mats revealed the even distribution of the biological component in the fibers. The use of these semi-synthetic nano/micro-fibrous scaffolds in tissue engineering will contribute significantly to the production of xeno-free tissue equivalents for humans.
Development of Three Dimensional and Functionally Designed Nanofibrous Scaffolds

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The current challenge in tissue engineering scaffold is to fabricate a scaffold for the distribution and growth of cells. Electrospun nanofiber sheet have been shown to mimic the structure of extracellular matrix (ECM). Nanofibers produced using electrospinning possess unique advantage that is similar to nano-scaled nonwoven fibrous structure of ECM. Although these nanofibers have shown great potential for use as tissue engineering scaffolds, a significant problem that affects tissue engineered electrospun nanofibrous scaffolds is poor infiltration of cells into the 3D structure. In this study, we prepared three different types of nanofiber scaffolds, PLGA/Gelatin/HAp composite nanofiber, three-dimensional nanofiber scaffold, and nanocylinders. Physical manipulation can enhance cellular infiltration into electrospun scaffolds. These three dimensional showed great promise for design of cell permeable nanofibrous scaffolds for tissue engineering applications. To fabricate three-dimensional nanofibrous scaffold, electrospun nanofiber was treated ultra-sonication. The porosity of electrospun nanofibers was highly enlarged by ultra-sonication in an aqueous solution. The cell infiltration potential was greatly increased with respect to an increase in pore size and porosity. These three dimensional nanofibrous scaffolds fabricated by an ultra-sonication process allowed cells to infiltrate easily into the scaffold. And, poly (L-lactic acid) (PLLA), a biodegradable and biocompatible polyester, was electrospun to produce nanofibers that were treated with an amino group containing base in order to fabricate polymeric nanocylinders. The aspect ratio of the PLLA nanocylinders was tunable by varying the aminolysis time and density of the amino group containing base. The effects of changes in nanofibrous morphology of the PLLA nanocylinders/macro-porous gelatin scaffolds on cell adhesion and proliferation were evaluated. These results indicate that the gelatin/PLLA nanocylinder composite is a promising way to fabricate 3D nanofibrous scaffolds that accelerates cell adhesion and proliferation for tissue engineering.
A novel spinning technology for manufacturing of fibers from hyaluronic acid and its derivatives has been developed by researchers of the company Contipro. The fibers have a form of endless monofilaments with a diameter between 60-100 microns. Single fibers would have only limited utilization in the medical area so that textile technologies have to be employed to form a fabric applicable as a medical device. Ring spinning technology has been modified for production of threads from several hyaluronan monofilaments and for their blending with fibers made from other biocompatible polymers like PAD or PLA. Different constructions of threads have been prepared and their mechanical properties have been tested. Technological trials have been carried out to assess whether the threads composed from hyaluronan based fibers withstand exposure to mechanical conditions during textile processing. Air humidity has been evaluated as a principal factor that influences processability of the threads. Using different kinds of weaving and knitting industrial machinery several fabric samples with different patterns have been successfully prepared. Textile form of hyaluronan together with its inherent bioactive properties open new opportunities of application as an implant or a material for regenerative medicine.
**In-Vitro Evaluation of Nanofibrous Scaffolds Modified by Inkjet Printing**

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Electrospinning as well as inkjet printing has emerged as useful tools for creating spatially organized materials for tissue engineering. Electrospun nanofibrous scaffolds are aimed to mimic the architecture and biological functions of the extra-cellular matrix (ECM). By incorporating conductive material (PANi) and bioactive particles (HAp) into an electrospun scaffold we have formed a bioactive, hybrid scaffold system, which provides an electrically conductive environment. Combination of electrospun, biodegradable scaffolds with conductive polymers in the future would enable us (by the use of controlled electrochemical signals) to stimulate a multitude of cellular functions. The aim of this work was to investigate the effect of inkjet printing on the properties of obtained scaffolds but also the effect of deposited conductive polymer on the attachment, proliferation, migration, and differentiation of bone cells. The nanocomposite scaffolds were characterized in terms of their porosity, mechanical properties and bioactivity, but also by in-vitro cellular tests using NHOst cells. Mineralization process and ALP activity were studied to estimate the cells differentiation.
Chitosan is a polysaccharide hydrogel appropriate for tissue engineering applications due to its intrinsic properties such as biocompatibility, biodegradability, antibacterial activity, wound healing properties and bioadhesive character. Nevertheless, the mechanical properties of chitosan in the hydrogel form can be insufficient for many applications. Thus, reinforcement with a stiff inorganic phase appears as an appealing strategy to prepare chitosan hybrids with enhanced mechanical properties. In this work, chitosan-silica hybrids were produced. Chitosan macroporous scaffolds were prepared by the freeze-gelation technique and the inorganic phase of silica was introduced inside the pores by sol-gel reactions in acidic medium. In order to stabilize chitosan and be insoluble in acidic media, chitosan was crosslinked with genipin. Different genipin/chitosan molar ratios were tested (0.01, 0.05, 0.1, 0.25 and 0.5) to find the minimum quantity of genipin required to form a network insoluble in acidic medium that was 0.05. Sol-gel reactions were performed using Tetraethyl orthosilicate (TEOS) and 3-glycidoxypropyltrimethoxysilane (GPTMS) as silica precursors. GPTMS served also as a coupling agent between the free amino groups of chitosan and the silica network. The morphology of the composite was analyzed by scanning electron microscopy showing that the silica phase appears as a layer covering the chitosan walls. The amount of silica was determined from the residual in thermogravimetric analysis and the mechanical properties of the hybrids were characterized by means of compressive stress-strain measurements while being immersed in water showing an increase of elastic modulus up to two orders of magnitude.
Tissue engineering is a promising approach for creating small diameter vessels by combining autologous vascular cells with a natural and/or synthetic scaffold. Among numerous techniques to fabricate vascular scaffolds, electrospinning has been widely used due to its ability to mimic features such as the morphological, chemical and mechanical characteristics of the native ECM. Collagen and elastin are the two major proteins of native artery, which provide tensile support and confer elasticity to the blood vessel. A successful scaffold should closely mimic the ECM of native blood vessels both mechanically and biologically. In this respective we utilized a synthetic polymer PU and a natural polymer gelatin to fabricate a scaffold for vascular tissue engineering.

Polyurethane is often used in blood-contacting devices due to their thrombo-resistance properties, elasticity and biocompatibility with tissue and blood. Therefore, we combined the excellent mechanical properties of a hydrophilic polyurethane named Tecophilic® (TP) with gelatin (Gel) that possess great cell affinity, by electrospinning blends of PU and gelatin with weight ratios of 70:30, 50:50 and 30:70 to develop a novel substrate for vascular tissue engineering. The morphological and mechanical properties of the electrospun scaffolds were studied using SEM and uniaxial tensile testing. We investigated the effect of different compositional features of the electrospun scaffolds towards the proliferation and morphology of human aortic SMCs by MTS assay and SEM, respectively. Increased the gelatin content within the composite PU/Gel scaffold resulted in higher cell proliferation, as confirmed by MTS assay and SEM micrographs, and lower elastic modulus. We suggest the potential application of electrospun PU/Gel with weight ratio of 50:50 as optimized compositions to adequately support vascular regeneration.
PCL-based Ternary Composite Scaffolds with Tunable Degradation Rate and Improved HBMC Response for Bone Tissue Engineering

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Due to limited number of FDA-approved biodegradable polymers and their thermal properties, poly(ε-caprolactone), PCL, is the best choice for application in fused deposition modeling process (FDM). However, this polymer exhibits very long degradation rate and relatively poor bioacceptance. The aim of this study was to tune change of mechanical properties of PCL-based composite scaffolds during degradation and evaluate bioacceptance of resulting 3D constructs.

Ternary composites containing PCL, bioactive filler and PLGAs with various compositions and molecular weights were used to fabricate scaffolds by means of FDM. The scaffolds were degraded in Simulated Body Fluid (SBF) for period up to 6 months. Compressive mechanical properties were measured by means of mechanical tester. Additionally, surface colonization (cell outgrowth from multicellular spheroids) and expression of bone-specific alkaline phosphatase (bALP) by human bone marrow mesenchymal stem cells (HBMC) were studied.

Change of mechanical properties of the ternary composite scaffolds during degradation could be tailored better by varying compositions of PLGAs than their concentrations and molecular weight. During first weeks of degradation increase of yield strength and Young's modulus of the scaffolds was observed. Subsequent deterioration of mechanical properties was gradual and the offset of their decrease ranged from 5 to 24 weeks. Additionally, surface colonized by HBMC was approximately 5x higher on the ternary composite than neat PCL scaffolds. These results show that addition of different PLGAs enables to tune change of mechanical properties during degradation and promotes colonization of the PCL-based porous scaffolds by HBMC.
To develop tissue engineering strategies useful for repairing damage in the central nervous system it is essential to control and optimize neural cells growth and interactions with functional scaffolds. The aim of this study is to develop an artificial scaffold of polylactic acid (PLA) nanofibers to induce an environment that mimic embryonic radial glia organization and favors neuronal migration after a brain injury. For this purpose uncoated 3D electrospun PLA nanofibers were used to study the behavior of neural cells from mice cerebral cortices in vitro. Both, random and aligned fibers supported neural cells growth, but only aligned fibers permit neural cells invasion. Moreover, aligned fibers induce immature phenotypes in neuronal and glial cell cultures. Glial cells grown in aligned fibers showed bipolar shape and expressed the radial glia markers Nestin and BLbP, and the progenitor marker Pax6. Neurons grown in aligned fibers were characterized by a decrease in the expression of post-mitotic neuronal marker β-III Tubulin, and an increase of neuron restricted progenitor marker, Tbr2.

On the other hand, lactate is the degradation product of PLA scaffolds. The degradation study showed a linear release of lactate to the medium in the physiological range of μM. In order to explain the role of lactate in cell phenotype, cultures were treated with L-Lactate. Lactate did not change glial phenotype, while neuronal cultures adopted an immature phenotype similar to when cultured on PLA, maintaining neuronal progenitors.

According to previous work, these results suggest that PLA properties might act synergistically with nanotopography in the modulation of the astrocytic phenotype, but probably is the lactate released by the scaffold which guides neuronal maturation and neuronal progenitor self renewing.
The Effect of Growth Factor Release from Nanostructured 3D Polymeric Constructs on Proliferation of Osteoblast Cells for Bone Tissue Engineering

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The electrospinning technique provides fibers ranging from a few nanometers to micrometers with large surface areas. The simplicity of the process makes it very attractive for tissue engineering applications. Poly-blend (mixtures of synthetic and natural polymers) nanofibers can mimic the tissue structure by providing surface features of native tissue that promote cell survival. In the current study, blends of poly-β-caprolactone, poly-l-lactic acid and gelatin polymers used to construct 3D scaffolds in a double-layer format. With the aid of a controlled release system, scaffolds were also made biologically active. Preliminary cell culture studies were performed with osteoblast cells using fibroblast growth factor-2 as the controlled release agent. Promising results were obtained in cell proliferation studies (MTS assay) using this multi-functional and double layered scaffold. The performance of the scaffold will now be tested in stem cell differentiation. The authors would like to thank the Scientific and Technological Research Council of Turkey (TUBITAK, Project No. 111M787) for providing financial support for a part of this project.
Evaluation of the Elasticity/Viscosity Ratio of Biocompatible Fibrin-Agarose Scaffolds


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Fibrin-Agarose (FA) hydrogels have previously shown usefulness as biocompatible scaffolds for the generation of bioengineered tissues. However, the biomechanical properties of these biomaterial still need to be improved by varying the agarose concentration and water content. The aim of this work was to evaluate the biomechanical properties of different FA scaffolds to identify the most suitable hydrogel types for use in tissue engineering. In this work, we generated different FA hydrogels with increasing agarose concentrations (0%, 0.1%, 0.2%, 0.3%, 0.4%, 0.5%) and hydration grades (80%, 90%, 99%) using nanostructuring methods. Rheological properties of all these hydrogels were evaluated by using a shear stress controlled rheometer using steady-state and dynamic measurements. In each scaffold, the shear modulus (G), elastic modulus (G') and loss modulus (G'') were determined. Steady-state measurements showed that the shear modulus increased at higher agarose concentrations, and decreased at higher hydration grades, suggesting that nanostructuration is able to reduce the water content and increase stiffness of the biomaterial. The highest shear modulus was obtained for FA scaffolds with an agarose concentration of 0.5%. Oscillatory measurements showed that the G' elastic modulus was always higher than the G'' modulus, suggesting that these scaffolds were more elastic than viscous. These results suggest that the agarose concentration and water content should be controlled when a stiffer biomaterial is required. This work was supported by grants FIS PI10-1582 and PI11-2668 from the Spanish Instituto de Salud Carlos III.
Osteoblastic Differentiation Effects of Electrospun Polycaprolactone (PCL)/Fucoidan (*Undaria Pinnatifida*) Nanofibres on Osteoblast Like Cell (MG-63) for Bone Tissue Regeneration

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In this study, we designed a new biocomposite comprising electrospun polycaprolactone (PCL)/fucoidan using various compositions (1, 2, and 10 wt.%) of fucoidan from *Undaria Pinnatifida*. The resultant electrospun composites exhibited improved tensile modulus and strength for limited weight fractions (<10 wt.%) of fucoidan when compared with the pure PCL fiber mats. In addition, the three (PCL)/fucoidan (1, 2, and 10 wt.%) promotes osteoblast differentiation by increasing expression of several osteoblast phenotype markers such as total protein content, alkaline phosphatase (ALP), osteocalcin, osteopontin and bone mineralization in human osteoblast like cell line (MG-63) in dependent compositions and compared with pure PCL mats. These observations suggest that fucoidan-supplemented biocomposites would make excellent materials for tissue-engineering applications.
Nanofiber Morphology Optimization for Using as Tubular Artificial Blood Vessels

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Electrospinning of polymer solution leads to the nanofibrous mats mimicking the structure of native extracellular matrix. The modification of electrospinning setup with a rotating mandrel collector results in deposition of nanofibers onto a tubular scaffold being used as vascular grafts. Polycaprolactone (PCL) is one of the most used polymers thanks to its excellent properties such as biocompatibility, biodegradability and mechanical strength. In this study, multilayer structure composed of PCL was designed for scaffold fabrication comparable to the structure of native vessels in human body. Inner layer of the scaffold is composed of thin fibers whereas the outer layer is formed by thick fibers in order to control the pore size for target cell type. Moreover, orientation of the fibers was investigated with adjusting the speed of rotating mandrel to improve cell adhesion. Electrospinning parameters for production of each layer were optimized and final vascular graft was mechanically tested.
Characterization and Comparison of Electrospun Polycaprolactone Based Composite Scaffolds for Osteogenic Differentiation of Human Adipose Stem Cells

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Electrospinning is considered as a promising scaffold fabrication method for bone tissue engineering, because non-woven membranes of electrospun fibers are thought to mimic the bone extracellular matrix. In the current study, we investigated the addition of hydroxyapatite (HA), natural component of bone, into the electrospun scaffolds and the potency of the scaffolds to modulate the osteogenic differentiation of human adipose stem cells (hASCs). Adipose tissue is an attractive and abundant source of multipotent stem cells, and hASCs have already shown their clinical relevancy in bone tissue engineering applications. Here, HA was combined with polycaprolactone (PCL) either during spinning resulting inside the fibers, or precipitated onto the PCL scaffold surface after spinning. Also blank PCL scaffold was used in comparison. The scaffolds were characterized by scanning electron microscopy (SEM), tensile test, and apatite forming capacity judged by SEM. The cell viability in the scaffolds was evaluated by Live/Dead staining and proliferation by CyQuant Assay. The osteogenic differentiation was analysed after 1-3 weeks culture by quantitative alkaline phosphatase activity assay (qALP) and immunological stainings by osteogenic markers, osteopontin and osteocalcin. Studies were conducted in a presence of basal growth medium and osteoinductive medium conditions. Our results indicated that HA on the scaffold surface supported best the attachment of cells, whereas the blank PCL scaffold was found to enhance the osteogenic differentiation most and carried also best mechanical properties. As a conclusion, HA may not improve the properties of electrospun PCL scaffolds for bone tissue engineering.
Bone Tissue Engineering Using Macroporous PHBV-ELP Scaffolds

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Elastin-like polymers (ELP) are genetically engineered proteins which contain cell recognition sequences such as RGD or REDV. In this study poly(3-hydroxybutyrate-co-3- hydroxyvalerate) (PHBV) scaffolds coated with ELP with REDV amino acid sequences (special for endothelial cells) were developed with wet spinning technique and tested their suitability for bone tissue engineering. PHBV was dissolved in chloroform (8%) and extruded into methanol to prepare scaffolds by using wet spinning. The surface activation of polymer was achieved by treatment with oxygen plasma (50 W, 5 min) and then the surface of polymer was coated with ELP via dip coating technique. The ultimate compressive strength of dry scaffolds under compression (5 mm/min) was 4.65±0.69 MPa which is very low for substituting native cortical bone (~100 MPa).1 The fiber diameter was around 90 μm and a porosity of 75% which was found by using SEM micrographs measured with NIH Image J program (Figure 1) and pycnometry. According to FTIR-ATR upon treatment with O2 plasma new peaks at around 1530 cm−1 and 1650 cm−1 was occured due to the stretching of carbonyl groups (Figure 2). After the ELP attachment, these peaks became more intense. This probably was related with the amide I and amide II groups of ELP attached onto the PHBV film. AFM and SEM micrographs indicated the roughness of the surface decreased upon oxygen plasma treatment from 343.4 nm to 160.0 nm but increased to 280.3 nm after ELP attachment.

Figure 1: SEM image of PHBV scaffold

Figure 2: FTIR spectra of PHBV film

The wet spun scaffold was sufficiently porous, had sufficient strength for non-load bearing bone defect applications.
Effects of Physical and Chemical Pre-Treatments on Degradation of Alginate and Alginate-Hydroxyapatite Composites

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Recently, injectable composites for bone regeneration have been developed based on the combination of ultra pure alginate as the matrix phase and hydroxyapatite as the dispersed phase. Since the human body lacks enzymes to degrade alginate macromolecules of high molecular weight, other strategies need to be considered to stimulate degradation of alginate and alginate-based composites. Therefore, we have investigated the influence of physical and chemical pre-treatments on the degradation behavior of alginate and alginate-hydroxyapatite composites. To this end, ultra-pure alginate was pre-treated by 1) γ-irradiation (20, 50 and 80 kGy), 2) autoclavation (at variable duration and number of cycles), and 3) (partial) oxidation (1 and 4%). The pre-treated alginate was analyzed by SEC-MALLS technique and NMR to evaluate the effects of treatment in the polymer structure. Afterwards the composite gels obtained from pre-treated alginate were evaluated regarding rheological properties, mass loss and calcium uptake after immersion in SBF solution. The results showed a decrease in molecular weight (Mw) for all the alginate pre-treatments with the highest decreased observed for the γ-irradiated and partially oxidized samples. The physical degradation of the composite samples was monitored upon immersion in SBF solution by rheology and mass loss. The results showed a strong decrease in storage modulus and mass for the composites obtained from the lower Mw alginate. Furthermore, the mineralization characteristics of the composites were followed by Calcium measurement in the SBF solution, which revealed that extent of mineralization increased with decreasing molecular weight.
**In-situ Adipose Derived Stem Cells Encapsulated in PEG-HA Hydrogel Scaffold as a Reservoir of growth Factors for Wound Healing**

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Chronic wounds have been hard to manage despite the advances in tissue engineering. Stem cells delivery has shown to be useful strategy. One approach is to deliver stem cells as reservoirs of growth factors from biomimetic hydrogels. In this study a PEGMEMA-PEGDA-HA hydrogel scaffold is synthesised via reversible addition fragmentation chain transfer (RAFT) polymerisation to create a biomimetic stem cell niche for human adipose derived stem cells. The hypothesis underlying this study is that PEG based hyperbranched copolymer synthesized via RAFT, in combination with hyaluronic acid (HA), can form a 3D scaffold to provide a stem cell niche upon *in situ* gelation to support and maintain stem cells and lead to secretion of growth factor for wound healing. PEGMEMA-PEGDA polymer was synthesised via RAFT. Hydrogels were formed by cross-linking hyperbranched PEG with thiolated hyaluronic acid. In-vitro analysis using human adipose derived stem cells (hADSCs) for viability, proliferation and secretion was performed for up to 14 days. Hydrogel induced secretion of angiogenic cytokines and expression of transcription factors associated with maintenance of pluripotency and self-renewal were studied. hADSCs seeded within the hydrogels can remain viable for up to two weeks *in vitro*, secreting growth factors (VEGF, bFGF, PI GF) associated with angiogenesis and maintaining stem cell markers after 7 days.

Our preliminary data suggests that HA-PEG hydrogel can act as a functional niche for maintaining hADSCs stemness, which is capable of enhancing hADSCs regenerative potential for wound healing.

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Embroidered and Surface Coated Polycaprolactone-co-Lactide Scaffolds - a Potential Graft for Bone Tissue Engineering

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This work presents a tissue engineering strategy using embroidered and surface coated polycaprolactone-co-lactide (trade name: PCL, Catgut GmbH, Germany) scaffolds as bone graft substitutes for large bone defects. The scaffold design and fabrication, the scaffolds properties as well as the surface modification and their influence in vitro were evaluated, followed by an in vivo analysis of the scaffold using orthotopic implantation models in rat (femur) and sheep (tibia) critical size defects (CSD). To create an artificial extra cellular matrix on the scaffold surface collagen I was immobilized, resulting in an enhanced cell attachment and proliferation of human mesenchymal stem cells (hMSC). An additional coating with chondroitin sulfate induced the osteogenic differentiation of hMSC in regular cultivation media without the common differentiation additives. To fill a CSD in long bone a 3 dimensional PCL scaffold with a pore size of 0.2-1 mm and a huge open and full interconnective porosity of 87 % was created. Rats and sheep showed new bone development to the point of bridging the critical size defect. The bone grew from the fracture ends along and into the scaffolds. Histological investigations showed a complete vascularized implant and new bone formation by direct and endochondral ossification. The present studies provide the first evidence that embroidered PCL scaffolds can act as temporary matrix for cell migration, proliferation and differentiation for bone tissue engineering applications and describes an alternative approach using biodegradable, embroidered, surface modified scaffolds for reconstruction of large bone defects.
Biodegradable Bone Replacement Material from Low Cytotoxic Monomers

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The fabrication of 3D scaffolds by lithography-based additive manufacturing technology (AMT) represents an appealing approach in bone tissue engineering. In this study, (meth)acrylates were replaced by vinyl esters and vinyl carbonates with exceptional low cytotoxicity. In vitro cytotoxicity studies with osteoblast-like cells proved that vinyl esters and vinyl carbonates are 1 order of magnitude less cytotoxic than methacrylates and 2-3 orders of magnitude less cytotoxic than acrylates. Photoreactivity of these monomers lies between those of acrylates and methacrylates that is sufficiently photoreactive for AMT. However, monomers containing abstractable hydrogens (e.g. ethylene glycol units) are even less photoreactive then methacrylates. By addition of thiols to these monomers, curing speed can reach the value for acrylates. Rates of degradation and mechanical properties can be tuned over a broad range. All polymers were significantly stiffer than PCL, being almost as stiff as PLA. Degradation of the polymers results in the formation of non-toxic FDA approved poly(vinylalcohol) of low molecular weight that can be easily transported within the human body. Finally, in vivo testing proofed a good biocompatibility of these materials. Obtained specimens featured abundant formation of new bone around and inside the 3D scaffolds, providing a high bone-to-implant contact.

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Over 800,000 people suffer tendon injuries worldwide per annum. Over 7% of repairs re-rupture and an estimated 25% achieve poor clinical outcome. A global consensus on the ideal tendon suture technique remains elusive despite a wealth of ex vivo and clinical studies. Computational analysis permits the study of detailed stress and deformation which can indicate initial repair failure, potential ischemia and poor healing. We employed the Finite Element (FE) method to investigate stress patterns in sutured tendon repair. The FE model is informed by and validated against ex vivo tensile testing of porcine flexor digitorum profundus tendon. FE models of Kessler, Savage, Tsuge and Cruciate sutures are presented which indicate the tendon repair providing the most favourable healing conditions. Furthermore, the model can be adapted to assess prototype techniques in the pursuit of an ideal tendon repair. Success of the model is underpinned by use of an appropriate description of tendon mechanical behaviour. As such, we discuss the merits of describing tendon as isotropic, orthotropic, linear elastic, or hyperelastic. Furthermore, we explore use of homogenisation which permits convenient description of the multi-scale hierarchy of tendon. A linear elastic orthotropic tendon description employing homogenization provides greater agreement with laboratory data than an isotropic model. Development and validation of a hyperelastic FE model will provide further insight into the suitability of different mathematical descriptions for tendon in this application. These descriptions of tendon mechanical behaviour are adaptable to other soft tissues and may be applied to wider surgical repair and scaffold design applications.

Finite Element model of Kessler suture repaired tendon using an isotropic linear elastic description for tendon. (a) Half Kessler model undergoing a suture traction of 1.6x10^6 N/m², showing stress in tendon. (b) Cut view of Kessler model, showing high stress in the anchor region of the Kessler repair. (Blue = low stress. Red = high stress.)
Peripheral nerves could be injured due to various reasons such as trauma, compression status, inflammatory disease, infections, surgical procedures etc [1]. The repair of peripheral nerve injuries with an autograft is still gold standard, although the method is associated with the size of the nerve gap and donor-site morbidity [2]. An alternative approach is to use an aligned polymeric nerve conduit to provide a guidance channel for the regenerating nerve [3]. But, collapse is still a problem for this approach. Here, we developed bilayer conduit, the inner layer having longitudinal aligned PHB nanofibers to promote nerve regeneration, and the outer layer having chitosan membrane for mechanical support. Inner aligned PHB layer was produced by electrospinning technique (Figure 1 A, C). The nanofibrillar scaffolds were prepared by using electrospinning 5 % (w/v) PHB solution was prepared in chloroform at 60°C. The polymer solution was then delivered to a 20-gauge metal needle (OD=0.91mm) connected to a high-voltage power supply. A CZE1000R Spellman (Spellman, USA) high voltage power supply was used to generate a fixed potential of 20kV. The polymer solutions were delivered at 2 ml/h flow rate by using Goldman syringe pump (Biasis, Turkey). The distance between the tip of the syringe and collector was 15 cm. A drum collector at 3100 rpm was used in the study (Figure 1B). Aligned PHB nanofibrous membrane was shaped into a tube by thermally. Internal diameter of the tube was 1 mm, the wall thickness was approximately 0.75 mm and length of the tube was 15mm (Figure 1 D, E). The morphological appearance of the nanofibrillar scaffolds was observed by scanning electron microscope (ZEISS EVO 50 EP, Germany) (Figure 1 F). 3 % chitosan (w/v) was dissolved in 1 % acetic acid solution and added 5 % glycerol (w/w, chitosan) to provide elasticity. The chitosan solution was applied onto the outer side of PHB tubes. Then, chitosan layer was cross-linked by 1% NaOH solution. The final grafts were washed several times with pH 7.2 PBS (Figure 1 F, G). This bilayer conduit is thought to has a great potential to be used as a nerve guide by adjusting the chitosan and crosslinker concentrations. The optimization studies for this purpose is still ongoing research in our group. Thus, this bilayer conduit is expected to increase the alignment of supporting cells and neurons to provide a guidance channel for the regenerating nerve.

Figure 1. A) Electrospin system, B) drum collector, C) produced aligned PHB nanofiber membrane, D and E) tube shaped aligned PHB nanofiber membrane; F) SEM image of aligned PHB nanofiber membrane; G) SEM images of chitosan covered PHB nanofiber membrane.
Biodegradable polymeric porous materials are used in tissue engineering as supports for cell transplant. In the case of bone regeneration or repair the stiffness of these materials is not high enough if the material must sustain mechanical loading in the site of implantation. On the other hand, non-functionalised polymers are not bioactive. Combination of polymers with hydroxyapatite or bioactive glass can improve significantly the properties of the supporting materials. In this work microporous polycaprolactone membranes were produced by freeze extraction technique that allows obtaining sheets with a network of well interconnected pores. The pore structure was filled with a silica precursor solution containing tetraethoxysilane, TEOS, with an acidic or basic catalyser and silica was produced by a sol-gel reaction. Microstructure of the inorganic phase was highly dependent on the composition of the silica precursor. Acid catalyzed sol-gel reaction yielded uniform silica coating of the pore walls. The continuity of the silica phase is proved when all the polymeric phase is eliminated by pyrolysis. On the other hand, basic catalyised reaction tends to produce silica nanoparticles loosely adhered to the PCL surfaces and they are easily delivered in aqueous media. In both cases the hybrid composites maintain the structure of open micropores. Mechanical properties of the hybrid composites were studied. Increased bioactivity was demonstrated by the deposition of biomimetic hydroxyapatite in immersion in simulated body fluid. The viability and osteoblastic differentiation of bone marrow rat mesenchymal stem cells cultured on the hybrid membranes was studied.
Electrospun Polymers for Tendon Repair: Polymer Chemistry Effect on Collagens Synthesis In Vitro

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Synthetic, degradable polymers are excellent candidates for rotator cuff repair patches, and electrospun polymer scaffolds made of polyglycolic-lactic acid (PGLA), poly-lactic acid (PLLA) and polydioxanone (PDO) have been previously evaluated as potential candidates for tendon repair. Here we present a comparative study of these in terms of in vitro matrix synthesis. This type of evaluation should assist in the selection of an appropriate polymer for the construction of tendon repair patches. Human tendon cells were extracted from rotator cuff tissue resected during surgical repair, with appropriate ethical approval. Randomly oriented nanofibre scaffolds with comparable fibre diameter from the different polymers were prepared using a single nozzle electrospinning set-up. Cells were grown on scaffolds for 21 days. RNA was extracted by Trizol homogenisation in a GentleMACS (Miltenyi Biotec). RNA samples were reversibly transcribed to cDNA and RT QPCR were performed using a ViiA7 (Life Technologies) with QuantiTect primer assays (QIAGEN). Results are in relative expression to GAPDH. Collagens I and VI were differentially expressed on different polymers, with higher expression noted on PDO scaffolds. β-actin expression was also affected. This could be due to the varying mechanical characteristics of the different polymers. There was no Collagen II expression on any of the scaffolds, indicating no chondrogenic phenotype. Collagen expression on the different scaffolds varied significantly. Whilst Collagen I (subunit 1) was expressed in low quantities, Collagen VI was highly expressed and may be more suitable as a marker for tendon matrix synthesis in vitro.

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Electrospun poly(L-lactide)/poly(ε-caprolactone) Blend Nanofibrous Scaffold: Characterization and Biocompatibility with Human Adipose-derived Stem Cells

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The essence of tissue engineering is the fabrication of autologous cells or induced stem cells in naturally derived or synthetic scaffolds to form specific tissue types. Polymers are thought as an appealing source of scaffold owing to the diversity of their physicochemical properties and can be electrospun into nano-size to mimic natural structure. Poly (L-lactic acid) (PLLA) and poly (ε-caprolactone) (PCL) are both excellent aliphatic polyester with almost “opposite” characteristics. The controlling combination of PLLA and PCL provides varying properties and makes diverse application. Compared with the copolymers of same components, PLLA/PCL blend demonstrates potential in regenerative medicine as a simple, efficient and scalable approach. In this study, we electrospun PLLA/PCL blend of different weight ratios into nanofibrous scaffold (NFS) and their properties were detected including morphology, porosity, degradation, ATR-FTIR analysis, stress-stain assay, and inflammatory reaction. To explore the biocompatibility of the NFS we synthesized, human adipose-derived stem cells (hASCs) were used to evaluate proliferation, attachment, viability and multi-lineage differentiation. In conclusion, PLLA/PCL blend fibers prepared by electrospinning with 3 weight ratios supported hASCs well and the NFS of 1/1 weight ratio showed better properties and cellular responses in all assessments, implying it a biomimetic scaffolds for tissue engineering.
Formation of Crimp Structures In Oriented PDO Electrospun Scaffolds: an Opportunity for Tendon Repair?

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Aligned nanofibrous electro spun scaffolds have been previously proposed as potential scaffolds for tendon repair, with emphasis on biomimetic design, postulated to encourage tissue regeneration (Hakimi et al., 2012). In this report, we investigate the interaction of primary tendon-derived cells with oriented electrospun polydioxanone (PDO) scaffolds with a crimp pattern. Aligned scaffolds were produced using a single nozzle electrospinning set-up with a rotating collector. To induce early crimp, scaffolds were incubated in ethanol 70% for 2 hours directly after the electrospinning process. Human tendon cells were extracted from rotator cuff tendon tissue obtained during surgical repair, with appropriate ethical approval. Cells were cultured on the scaffolds for at least 14 days. A regular crimp pattern was induced within the scaffolds, as shown in Figure 1. The characteristics of the crimp are very similar to what is observed in native tendons. Human tenocytes grown on the nanofibrous scaffolds follow the crimp pattern, resulting in tendon-like tissue constructs. The PDO scaffolds were fabricated and oriented with a crimp morphology which is similar to the appearance of native tendon. With these biomimetic features, this novel PDO scaffolds stand as excellent candidate materials to support tendon repair.

![Figure 1: Oriented electrospun PDO scaffolds displaying their regular crimp pattern under scanning electron microscopy (a). Tenocytes grown on the crimp scaffold are shown in (b): red = cells, blue = scaffold.](image)

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New Derivative of Hyaluronic Acid – Synthesis and Study of Chemical and Biological Properties

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Hyaluronic acid (HA) is a natural linear heteropolysaccharide consisting of beta-(1-4)-D-sodium glucuronate -beta-(1-3)-N-acetylglucosamine units, with a molecular weight between 5 - 13000kDa. This polymer is found at high concentrations in extra-cellular matrix, vitreous humour and skin. HA modified with an aldehyde group (HA-CHO or HA-aldehyde) has been extensively used for various medical and tissue engineering applications. The main advantage of the aldehyde moieties is their ability to react with a wide range of amino compounds at physiological conditions. Reaction of aldehydes with primary amines in water is reversible and this equilibrium is thoroughly shifted towards starting aldehyde and amine. This presentation would like to introduce an unique modification of HA: alpha,beta-unsaturated-6(GlcNAc)-oxo HA. This modification allows to immobilize primary amines more effectively than known saturated HA-aldehydes. Higher hydrolytic stability is due to conjugation of imine with –C=C- double bond. Two strategies for preparation of unsaturated HA-aldehyde were successfully tested and the chemical structure has been studied in details. In this study, the synthesis of new unsaturated HA derivate and its ability to attach amino compounds were examined. Prepared compounds did not have any effect on cell viability compared to the untreated control and is safety up to 1mg/ml. Cross-linked materials prepared from this precursor are biocompatible and suitable for applications in tissue engineering and regenerative medicine.
Microalgae have been proposed as an interesting, easy-cultured, infinite marine source in the use for natural functional ingredients, and several researches have been reported the possibility of finding novel bioactive compounds in these organisms. Especially *Phaeodactylum tricornutum* has high content of fatty acids such as EPA and DHA. These fatty acids have anti-microbial activities. Gelatin is an attractive natural polymer for biomedical applications due to its excellent biocompatibility and degradability into non-toxic products. The aim of this study is to input functionality of the *Phaeodactylum tricornutum* into gelatin nanofiber-based scaffolds. Especially, the anti-microbial activity of *Phaeodactylum tricornutum*, since preventing infection during wound closure is important. In this study, we prepared electrospun gelatin nanofibers containing *Phaeodactylum tricornutum* extracts by electrospinning in the form of 2D nonwoven mat. We examined the effect of *Phaeodactylum tricornutum* content on the spinability and the size of nanofiber. We also have done preliminary studies on the physiological effect of *Phaeodactylum tricornutum* incorporated nanofiber scaffolds. Solutions of *Phaeodactylum tricornutum* extracts and gelatin(type A) were prepared at different concentrations and relative ratios using TFE as solvent. We prepared well defined electrospun nanofibers containing *Phaeodactylum tricornutum* with diameters around 200nm. The nonwoven mat exhibited anti-microbial activity to MRSA. The wound closure rate were similar to the commercial wound dressing.
Surf{ace} Functionalization of Electro-spun poly(L)Lactic Acid Scaffolds with Heparin to Improve Tissue Integration for Surgical Treatment of Pelvic Organ Prolapse

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Better biomaterials are needed to treat pelvic organ prolapse because the current treatments have a high failure rate. Hence, biomaterials can be specifically functionalised to improve their integration within the host. The aim of this study is to improve biointegration of electro-spun PolyLactic Acid (PLA) scaffolds (Fig.1) to improve angiogenesis post-implantation. Electrospun PLA scaffolds were plasma polymerized with PolyAcrylic Acid (PAA) and coated with alternative layers of PolyEthyleneImine (PEI) and PAA or PEI and Heparin for a total of seven layers, in a layer-by-layer (LBL) coating approach. Coated scaffolds were dipped in heparin solution, dried and immersed in Vascular Endothelial Growth Factor (VEGF) solution. Surface chemistry was verified by X-Ray Photon Electron Spectroscopy. ELISA was used to quantify the amount of VEGF bound onto the scaffolds. XPS showed that plasma polymerization of the scaffold with PAA was successful. Heparin bound well to LBL-coated scaffolds, compared to non-functionalised scaffolds, showing an increase in VEGF binding. Heparin binding to electro-spun PLLA scaffolds is greatly improved by plasma polymerization and layer-by-layer coating, due to an increase in surface charge. This results in VEGF bound to the fibres of the scaffold and future work will involve testing the pro-angiogenic effect of the heparin-coated scaffolds both in vitro and in vivo.
Engineering Gelatin-methacrylate/poly(lactide-co-ethylene oxide fumarate) IPN Injectable Hydrogels for Bone Repair

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In modern approaches for bone repair and regeneration, osteogenic cells are suspended within a polymeric precursor solution and the resulted cell suspension is gelled in situ through different crosslinking schemes such as photo-crosslinking to provide a physical platform for encapsulated cells to proliferate. Photo-crosslinkable, naturally derived polymers such as gelatin-methacrylate (GelMa) are the material of choice for various tissue engineering applications due to their superior biological properties. Their application in load bearing tissues such as bone regeneration however is limited due to their low mechanical strength. In this study to address this problem, interpenetrating polymer network (IPN) hydrogel of GelMa/poly(lactide-co-ethylene oxide fumarate) (PLEOF) was fabricated, which the entanglement of PLEOF polymer network mechanically strengthen the GelMa hydrogel. UV light and Irgacure 2959 as the photo-initiator reagent were used to induce selfcrosslinking of GelMa and crosslinking of PLEOF polymer by using polyethylene glycol diacrylate (PEG-da) as the crosslinking agent. The gelation time and mechanical strength of hydrogels were optimized by tuning the concentration of GelMa, PLEOF and crosslinking agents. The hydrogel with the gelatine time of 1.5 minutes was prepared when using 100 mg/ml polyethylene glycol diacrylate. In addition, the Young’s modulus of IPN hydrogel with composition of 1:3:1 (GelMa:PLEOF:PEG-dama ratio) was 220 ± 12 kPa that was nine-fold higher than of neat GelMa hydrogel. This Young’s modulus was in the acceptable range for in vivo bone repair. The IPN hydrogels exhibited highly porous structure with average pore size of 170 ± 12 μm and porosity of approximately 87% which can provide a favourable platform for 3D cell proliferation. Human osteoblast (HOB) cells exhibited 40% higher metabolic activity within IPN hydrogel compared to neat PLEOF hydrogel after 7 days of in vitro culture. GelMa/PLEOF hydrogels are deemed to be potential candidate as physical support for bone regeneration and repair due to their favourable, gelation behaviour, and mechanical and biological properties.
Ion Implantation on Electrospun Fabricated Poly (L-lactide/Caprolactone) (PLC) Nanofibers for Cell Proliferation in Tissue Scaffolds

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The use of polymeric materials produced by the electrospinning technique has gained considerable interest for tissue engineering applications. Ion beam modification of material’s surfaces is an alternative technique for improving the surface properties of polymeric materials for tissue engineering applications. In this study, for enhancing cell adhesion and proliferation, Poly (L-lactide/Caprolactone) 70/30 (PLC) nanofibrous scaffolds were fabricated by an electrospinning process and implanted by Au and Au+O ions. In the first part of this work, nanofibers were produced by electrospinning with a voltage of 20kV and flow rate of 1.5 mL/h, and deposited on a drum which was rotating with 1500 rpm and at a distance 12 cm from the spinneret tip for aligned nanofiber production. In the second part of this work, PLC nanofibers were implanted with Au and Au+O ions with fluencies of 1x10¹⁴, 1x10¹⁵, 1x10¹⁶ ion/cm² and extraction voltages of 20kV, 30kV. Finally, cell culture tests were performed on Au and Au+O implanted PLC electrospun samples and the results compared with unimplanted ones. Samples were incubated with human neuroblastoma cells for 2 and 4 days in a culture medium at 37 0C, and viewed with Scanning Electron Microscope (SEM) micrographs and Fluorescence Microscopes. The wettability of implanted and unimplanted surfaces were evaluated by contact angle tests. Implanted and unimplanted samples were analyzed by X-ray photoelectron spectroscopy (XPS) for surface chemical characterisation. XPS results were consistent with TOF result. Results of our study suggested the potential application of implanted PLC nanofibrous scaffold was ideal substrates for nerve tissue engineering.
Biocompatibility of Electrospun Produced Vacular Grafts and Modification of their Stiffness with Electron-beam Irradiation

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Vascular grafts were fabricated via electrospinning of polycapro lactone (PCL), nylon 6 and poly(lactic-co-glicolic acid) (PLGA) solutions in 1,1,1,3,3,3-hexafluoro-2-propanol. Minimal concentrations of gelatin and elastin in the basic polymers solutions sufficient for supporting of endotheliocytes adhesion and proliferation were found. Influence of the 3D matrixes to induction of inflammation was studied using primary endotheliocytes, fibroblasts and blood nucleated cells model by measuring of proinflammatory cytokines level. Influence of the polymer composition and electrospinning conditions onto graft-induced platelets aggregation and hemostasis was evaluated using standard blood tests using Chrono-Log 540- VS and Sysmex CS-2100i analyzers. It was shown by ESM that the grafts consist of approx. 1 micron fibers of polymers and possesses reasonable tensile strength and Young’s modulus about 500 N/cm². In contrast to fibroblasts endotheliocytes were shown to be much more selective to polymers composition: the data of cell viability and proliferation using ethynyl deoxyuridine incorporation test demonstrate PCL and PLGA as the most convenient basic polymers. All matrixes tested do not contain LPS in the concentration sufficient for induction of TLR4 dependent immune response. The verified data on the influence of electrospun graft onto immune response and hemostasis will be presented at the conference. To check application of electron beam bremsstrahlung for sterilization of the implants and to increase stiffness of the grafts introducing stiffeners preventing occlusion and aneurisms, vascular grafts were treated with electron beam generated by ILU-6 accelerator producing 2 MeV electrons and exposing dose varied from 0 to 250 kGy with increment 25 kGy. It was shown that the dosage up to 25 kGy does not interfere with mechanical properties of the electrospun 3D matrixes while dosages from 50 kGy to 100 kGy increase rigidity of the material with subsequent destruction of the materials in dosages higher than 150-200 kGy.
The Regulation of the Osteogenic Capability of Electrospun Scaffold with Nanohydroxyapatite Filler

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In this study we fabricated novel electrospun PLDL/n-HAp composite scaffolds for bone tissue engineering. To investigate the effects of electrospun PLDL/n-HAp scaffold microstructure and chemical composition on cells attachment, proliferation and their morphology we conducted a series of in vitro tests. In particular, microstructure, porosity, filler distribution and concentration were examined using SEM, FTIR and WAXD. Furthermore, we investigated the formation of biomimetic calcium phosphate on their surface that indicates bone bonding capability. In vitro experiments using NHbSt osteoblast bone cells showed that the incorporation of n-HAp significantly improved cell attachment and upregulated cells proliferation and osteoblastic differentiation. It was found that cell attachment and differentiation was directly related to the scaffold microenvironment (i.e. structure and chemistry). The results demonstrated that the addition of nano-hydroxyapatite provided chemical cues that was a key factor that regulated osteoblastic differentiation. This study formed a strong foundation to design osteogenic scaffolds for bone tissue regeneration.
Cell-instructive Matrices for the Assembly of Vascular Structures

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The main objective of this research is to develop biomimetic scaffolds that can be functionalized with growth factors to guide angiogenesis and maintain vascular structures. We have established a PEG-based hydrogel platform for the orthogonal incorporation of multiple angiogenic growth factors and the creation of 3D microenvironment, by producing a set of different affinity linkers that can be specifically crosslinked inside the PEG scaffold. These linkers allow us to immobilize well-known angiogenic growth factors such as VEGF, PDGF and angiopoietin. We believe that cell-guiding growth factors are crucial for the maintenance of vascular structures and thus seek to incorporate ephrin ligands for the control of cell migration inside our scaffolds. We have therefore studied the effects of ephrins on developing blood vessels using the ex ovo chicken chorioallantoic membrane (CAM) assay. We are currently designing the PEG scaffolds by using 3D printing technologies to create controlled microenvironments. 3D printing will also be used for the placement of vascular cell types for the formation of tube-like structures. PEG scaffolds are largely devoid of biological functions, which makes them an ideal candidate for the development of the biomimetic matrices. However, our technology has the advantage that it is also transferable to fibrin matrices if needed. The final objective of this project is to develop pre-vascularized implants that will be able to rapidly connect to surrounding vessels after transplantation, resulting in the maturation and stabilization of the preformed vascular network.
Chitosan has been studied for various practical applications because of functions such as biodegradability, low toxicity, and acceleration of fibroblast formation in animal body, acceleration blood clotting, drug delivery, antimicrobial activity and high solubility in water. In this study, we attempted to prepare and characterize the chitosan membrane surfaces induced by metal–gas (MEVVA) ion implantation. Chitosan membranes were prepared in two different microstructures to investigate the structure effects on the protein adsorption and in vitro degradation. Dense and asymmetric chitosan membranes prepared by dissolving in acetic acid solution. For dense membrane production, solvent casting method was used. For asymmetric membrane preparation dry/wet phase separation method was used by using 20 minutes pre treatment time. By changing this time pore size and thickness of the membrane is changed that also effects the membrane properties like diffusion ratio, water absorption, degradation time etc. Chitosan membranes then were implanted by C and C+N ions by using MEVVA ion implanter with $10^{16}$ cm$^{-2}$ dose, and 1 pps frequency, 20 kV acceleration voltage. As a result of these, we investigated the effect of ion implantation on the protein adsorption behavior and in vitro degradation of chitosan films before and after the ion implantation. The chitosan films were prepared by solvent casting method for dense films, and dry/wet phase separation method is used to obtain asymmetric chitosan membranes. Characterization studies of these membranes were performed by using Scanning Electron Microscopy (SEM), Fourier Transform Infrared Spectroscopy (FTIR), and Differential Scanning Calorimetry (DSC). The ion implantation effect on the contact angle changes, water absorption, protein adsorption, biodegradation mechanism and neural cell attachment (B35) were also examined.
Nonthrombogenic Clopidogrel Modified Silk Fibroin Scaffold

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In this study, the silk fibroin/clopidogrel scaffolds were modified by crosslinking N, N′-methylene-di-acrylamide crosslinker with freeze drying technique at two different temperatures (-20°C and -80°C). Morphology, biodegradability, blood compatibility were characterized by means of scanning electron microscope (SEM), Fourier Transform Infrared spectra (FTIR) and thrombin time (TT). The results showed that the clopidogrel was successfully introduced to the silk fibroin scaffolds. The swelling percent of scaffolds prepared at -20°C was less than the swelling percent of the scaffolds prepared at -80°C. The pore sizes were greater as the freezing temperature during preparation process increased. The biodegradability studies of silk fibroin/clopidogrel scaffolds with protease enzyme showed that products were biodegradable with enzymes. The thrombin time of the product was much higher than those of the pure silk fibroin scaffold. The results indicated that the clopidogrel modified silk fibroin scaffolds could be considered as ideal candidates for tissue engineering applications.
Cross-linked Polyvinylpyrrolidone Nanocapsules as a Controlled Drug Delivery System

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Polyvinylpyrrolidone (PVP) is an ideal polymer for medical applications due to its biocompatibility and biodegradability. It is as well good polymer for nanofibrous production by electrospinning or for production of capsules by electrospraying process. Crosslinking of PVP is possible by UV radiation and by the setting of the time of irradiation one can adjust the degradation of PVP in the range from minutes to days or weeks. The size of capsules can be controlled as well in the scale from several microns down to submicron particles. Such a system can be used for precise drug delivery system or for designing of special scaffolds for tissue engineering with encapsulated proliferation factors. This is crucial for cell proliferation into the three dimensional scaffolds. Both electrospinning and electrospraying processes were designed for mass production by the using of needleless technology. Capsules with the solid content can be produced by electrospraying from polymeric dispersion with controlled parameters however capsules with the liquid agent can be prepared by the coaxial electrospraying. All mentioned techniques enable us to produce unique three dimensional scaffolds as well as intelligent wound covers.
A biomimetic approach to prepare bone grafting material comparable to natural bone, in terms of structure and composition, has attracted the attention of many researchers. We attempted to mimic collagen fibrils bearing apatite crystals in natural bone using gelatin, carboxylic acid functionalized carbon nanotubes (f-CNTs) and hydroxyl apatite (HA). We covalently grafted gelatin molecules on the surface of f-CNTs via the formation of amide linkages. HA crystals then were assembled on the gelatin-grafted f-CNTs in SBF solution, showing a multilayered core-shell structure consisting of f-CNT core and gelatin-HA shells (as a fibrous multilayered f-CNT/Gel/HA nanohybrid) in a similar constitution to that of the collagen fibers of natural bone. Mechanical properties (the tensile strength, the elastic modulus, and the elongation rate) of the new hybrid material showed a significant increase as compared to a pure (f-CNT free) gelatin and a physical mixture of f-CNT and gelatin: respectively 4.6~8.8, 9~11, and 28~42 times. The cell viability studies on the f-CNT/Gel/HA nanohybrid also suggested commendable biocompatibility, as compared to pure gelatin. The remarkable improvement in mechanical properties in tandem with other biological features observed supports the potential usefulness of the f-CNT, gelatin, HA-multilayered core-shell nanohybrids for the repair and regeneration of hard tissues.
Biomimetic properties of nano-fibrous electrospun scaffolds provide a great environment for tissue regeneration. Structural features such as fibre arrangement and orientation could potentially modulate the mechanical properties of the electrospun scaffold. This is particularly important in fabrication of scaffolds that are used to encourage regeneration in load bearing tissues. This study aims to study the effects of fibre arrangement on mechanical properties of electrospun scaffolds produced from two commonly used biodegradable polymers, polydioxanone (PDO) and polycaprolactone (PCL). Scaffolds with 3 different arrangements, Aligned, Dual (combination of random and aligned) and random, were produced using a single nozzle electrospinning set-up. For each type of scaffold, 5 separate specimens were used, measuring 50 mm in length and 5 mm in width. Specimens were tested to failure in tension using Zwick machine at rate of 0.5 mm/min until failure. We assessed thickness (mm), maximum failure load (N), ultimate strength (MPa), breaking strain (%) and Young’s modulus (MPa). Mechanical evaluation revealed the significant effect of fibre arrangement on the mechanical properties of electrospun scaffolds.

Figure 1: Mechanical characterization of scaffolds with aligned, dual and random fibre arrangement. A) There was no significant difference between the thicknesses of scaffolds with different fibre arrangements in both polymers B) Both aligned PDO & PCL scaffolds showed higher max failure load in comparison to other groups C & D) comparison of Strain failure (%) and Young’s modulus (MPa) of all three fibre arrangements in both PDO and PCL scaffolds.
Burns cause injuries affecting integrity of the skin. Treatments for this kind of injuries in Mexico are expensive and inaccessible for most of the population. Tissue Engineering is an alternative for tissue regeneration through the use of scaffolds made of biomaterials. Chitosan is a polysaccharide derived from chitin, while PLA is aliphatic polyester; both materials are biocompatible and biodegradable, and they can be used as scaffolds with biomedical applications. Our goal was to evaluate the biocompatibility of a scaffold made in the laboratory, which may improve the healing process of burn patients. Foreskin samples were obtained from consenting patients between 4 and 7 years old. Chitosan-PLA film was elaborated with chitin derived of shrimp. Fibroblasts were isolated using collagenase. Cells were seeded onto a scaffold and cultured under standard conditions. After 5, 10 and 15 days of culture, we evaluated viability with calcein and ethidium probe and cell attachment was analyzed with a Scanning Electron Microscopy. Cells were viable onto the scaffold at different time points. At day 5, fibroblasts remained over the film; however, some cells were round shaped. For days 10 and 15, fibroblasts had an elongated morphology, characteristic of its cellular type. At day 15, a monolayer covering most of the scaffold was observed. Micrographs showed round fibroblasts with pseudopods spreading over the surface at the initial days, and after days 10 and 15 there was cell attachment and fibroblasts covered the scaffold. Film based on Chitosan-PLA is a biocompatible scaffold that meets the conditions to maintain cell viability and cell spreading on its surface, suggesting that it could be a good candidate for the development of dermal substitutes.
Human Bone Marrow Mesenchymal Stem Cells’ Response on Precise-Geometry Controlled Scaffolds for Bone Tissue Engineering

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Engineering artificial scaffolds that enhance cell adhesion and growth in three dimensions is essential for successful bone tissue engineering. However, the fabrication of three-dimensional (3D) tissue scaffolds with highly precise geometry exhibiting complex micro- and nano-features still remains a challenge. Here, we report on the adhesion and growth of bone marrow mesenchymal stem cells (MSCs) on high resolution 3D scaffolds, which are fabricated using a hybrid organic-inorganic material by direct laser writing (DLW). We investigate the cellular response on the scaffolds in various time points. Furthermore, we explore the potential of the fabricated 3D scaffolds in bone tissue engineering. Our results indicate a novel organic-inorganic composite material, which can be structured into 3D complex-geometry scaffolds, and display a high initial cell attachment and promote cell growth. The strong initial adhesion and proliferation of bone marrow MSCs on the 3D structured composite exhibit the high potential of the material for use in scaffolds for bone tissue repair. In future experiments we plan to immobilize the osteoinductive growth factor BMP-2 on the material and explore the osteogenic response both in vitro and in vivo.
Assembling Modular Components to Engineer Articular Cartilage

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Engineering of complex three-dimensional (3D) tissues requires meeting a set of challenging scaffold design and cellular criteria. Many of these criteria are interdependent, making optimisation of individual aspects difficult. Adopting a modular approach, where the tissue is reduced to several components which can be separately optimised, offers opportunities to uncouple scaffold and cellular/extracellular matrix components. In this study, the use of modular microtissue assembly for producing engineered articular cartilage was investigated in vitro. Cartilage microtissues were placed within a 3D-printed scaffold, forming a tissue composed from two modular components. Fabrication, 3D distribution, and fusion of microtissues were investigated, as was the influence of cell-scaffold interactions and mechanical properties. Subsequently, optimised tissues and scaffolds were assembled and chondrogenic capacity of the construct characterised. Microtissues that induced chondrogenic redifferentiation could be mass-produced and selectively placed within the 3D scaffold. A coherent in vitro cartilage construct was assembled that stimulated hyaline-like matrix formation, as well as exhibiting dynamic mechanical properties similar to that of native cartilage. We show here that modular components can be individually tuned to enhance matrix formation, ease of biofabrication, mechanical properties, integration, and tissue unit fusion. This demonstrates advantages for articular cartilage tissue engineering where components can be separated, allowing a scaffold of appropriate mechanical properties to be combined with high-quality tissue units.
Dynamic Seeding of Rat Mesenchymal Stem Cells in Hydroxyapatite/Gelatin Robocasted Scaffolds

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A key issue in tissue engineering is the selection of a scaffold material that provides the specific conditions to direct the differentiation of mesenchymal stem cells (MSCs) into the desired phenotype. Hydroxyapatite (HA) is a promising material to fabricate scaffolds for bone regeneration. The present work explores the fabrication of low-temperature HA/gelatin scaffolds by robocasting and their performance in dynamic culture of MSCs. A low-temperature self-setting ink was developed, consisting of a suspension of alpha tricalcium phosphate (\(\alpha\)-TCP) powder in a 10 wt.% B-type gelatin solution, at a liquid to powder ratio of 0.65 ml/g. Cubic scaffolds were robocasted following an orthogonal pattern layer using a rapid prototyping machine with tips of 0.84 mm of diameter. The scaffolds were immersed in water for 7 d in order to allow for the hydrolysis of the \(\alpha\)-TCP to HA, with the subsequent improvement of the mechanical properties. Afterwards, 1 million of rat-MSCs were seeded in oscillation at 10 mm/min during 3 h inside a perfusion bioreactor system. The final composition of the HA/gelatin robocasted scaffolds mimics the composite structure of bone tissue. Besides, the compressive strength was in the range of trabecular bone (9.4 ± 1.6 MPa). A totally interconnected pore network of 500 µm in the printing plane and 300 µm vertically was obtained in the scaffolds, with a microstructure formed by a microporous entangled network of needle-shaped HA crystals. As can be seen in the figure, the scaffolds were suitable for dynamic seeding of rat-MSCs. Gelatin not only allowed to print and stabilize the printed scaffold until complete hardening of the structure, but also enhanced cell adhesion during dynamic seeding of MSCs.

Figure 1: Overall view of HA/gelatin robocasted scaffold, cells attached during dynamic seeding, and view of the macro and microstructure of the scaffold.
Effect of Pore Shape Gradient on human Mesenchymal Stem Cell Behavior in 3D scaffolds

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The aim of this study was to create 3D scaffolds presenting an axial gradient in pore shape by varying the plotting pattern, and evaluate its influence on human mesenchymal stem cells (hMSCs) growth and differentiation. PEOT/PBT copolymer 300/55/45 (PolyVation) scaffolds with (G) or without (NG) pore gradients were fabricated by rapid prototyping (RP). Fiber deposition pattern was kept constant at 0-90 and 0-15 for NG scaffolds whereas G scaffolds were prepared by changing the fiber deposition pattern every 7 layers (0-90, 0-45, 0-30, 0-15). The resulting scaffold presented pores with a squared shape on the bottom, which changed into a more and more rhomboid shape when moving along the Z axis. This corresponded to an increase in the major and a decrease in the minor diagonal length (Figure 1). Bone marrow derived hMSCs were seeded on the scaffolds (500,000 cells/scaffold). After expansion for 7 days in proliferation media, hMSCs were further cultured in basic, osteogenic and chondrogenic media for 1 and 28 days (8 and 35 days from seeding). Cell growth and differentiation were evaluated by DNA and ALP assays. Cell adhesion and morphology, and extra cellular matrix (ECM) formation were also evaluated by scanning electron microscopy (SEM).

Figure 1: The plot shows the increased cell seeding efficiency of the G scaffold. In the table on the right the change of the pore shape along the z axis is displayed.

Scaffolds presenting a gradient in pore shape showed a higher seeding efficiency of hMSCs. This could be due to an increase in number of fiber contacts and a correspondent sieve effect. hMSCs osteogenic and chondrogenic differentiation were not affected by the gradient. Further studies will aim at corroborating these findings with different donors.
Micro-robotic Assembly of Microgels for Regenerative Medicine

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Multiple methods such as acoustics, multi-layer photo-crosslinking, magnetics, nanoratchets, capillary forces, and microfluidics have been utilized to assemble cell-encapsulating hydrogels into tissue constructs via a bottom-up 3-D assembly approach. There is an unmet need to fabricate more complex and precise heterogeneous tissue constructs. Precisely pushing cell-encapsulating hydrogels into target locations using a micro-robot can enable a higher level of control over assembly process to achieve such complex final constructs. Here, we present magnetic micro-robotic assembly of cell-encapsulating hydrogels at scales of many hundreds of microns on a side into complex tissue constructs (Figure 1). Permanent magnet micro-robot motion is achieved by computer-controlled magnetic fields using visual feedback through a microscope, and can provide manipulation precision at the order of 10 micrometers. Mobile micro-robots can be remotely actuated for specific tasks in any fluid or air environment, without requiring a specialized surface. This manipulation and assembly approach can find broad applications in multiple fields including biosensing, tissue engineering, micro-physiological system engineering, and drug delivery research.

Figure 1: Assembly of PEG hydrogels by magnetic micro-robot
Bioinspired Microfabricated Scaffolds for Tendon Tissue Engineering

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Tendons are composed of packed collagen fiber bundles aligned parallel to the longitudinal tendon axis and surrounded by a sheath¹ that improves friction-free gliding. This study aims to bioengineer fiber bundled scaffolds to promote an aligned orientation of tendon cells as in native functional tissue. Moreover, these scaffolds were embedded in agarose to resemble tendon sheath or in a platelet lysate (PL)-rich agarose coating. PLs were chosen as a natural source of growth factors known to promote tissue regeneration². Fiber scaffolds made of a blend of starch and polycaprolactone (SPCL) were designed by 3D printing and rolled into bundle-like structures. Tendon cells obtained from surgery surplus were expanded in basic medium. 24h after seeding (2x10⁵ cells/scaffold) constructs were dip-coated in an agarose solution (2% in PBS) or in a PL (5mg/mL)-agarose solution for 7 days. Constructs were evaluated for cell morphology (SEM), metabolic activity (MTS), and Tenascin C (TNC) and Scleraxis (SCXA) protein expression by immunolocalization. 3D fiber bundle-like scaffolds were successfully tailored. The microfabricated structure allowed cell adhesion and promoted an aligned pattern on cell distribution. Although SCXA was minimally detected in the constructs, TNC was observed with higher intensity on agarose-coated constructs. The PLs enrichment of the scaffolds did not show a significant effect on cell morphology or on extracellular matrix (ECM) production. However, PLs are likely to play an important role in in vivo models. The developed strategy inspires scaffold interaction with ECM, being of utmost importance for functional tendon regeneration.

Dynamic self-assembly (DSA) or self-disassembly of building blocks into or apart from a structure-ordered and functionalized non-equilibrium thermodynamic system is one of the fundamental ways for organisms to organize and regenerate themselves. However, there have been no demonstrations of bottom-up tissue engineering using DSA. Herein, we demonstrate, for the first time, a prototypical DSA system for bottom-up tissue engineering. Faraday waves are explored as a reconfigurable and scalable template, which enables spontaneous patterning dispersed cell-encapsulating hydrogel units into a symmetric and ordered structure (e.g., ring shape, cross shape, Celtic shape etc.) at the air–liquid interface within a few seconds. The assembled structure can be reproducibly reconfigured into diverse sets of patterns by changing the distribution of Faraday waves in the liquid-carrier chamber. Further tissue culture was enabled by ultraviolet crosslinking and fixation of the assembled hydrogel units. Both cell viability and proliferation assays indicate that this method is biocompatible. Faraday wave directed self-assembly can generate 10X more types of structures with a 10 to 100X higher efficiency and speed comparing to previous static self-assembly methods in tissue engineering. In addition, our method exhibits some unique functionalities of non-equilibrium thermodynamic system, such as self-healing and self-adaptation, which widely exists in organisms. In general, DSA provide a new avenue to bottom-up tissue engineering, and Faraday wave based DSA can serve as a model system to enhance the understanding of biological systems.
3D Plotting of Fibrillar Collagen to Fabricate Scaffolds of Defined Architecture

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The rapid prototyping technique of 3D plotting allows the processing of a wide variety of synthetic and natural materials in form of slurries, highly viscous solutions, suspensions, melts and blends. The mild process conditions enable the application of biopolymers such as collagen and even the integration of sensitive components like growth factors or cells. We have fabricated porous scaffolds of defined architecture by plotting suspensions of fibrillar collagen type I, obtained from porcine skin. At low pH values, the pasty collagen material was plottable without additives to generate pure collagen scaffolds which were subsequently stabilized by chemical crosslinking. At neutral pH, the collagen suspension was processible by plotting if blended with hydrogels which were able to counter the liquid segregation during the plotting process. The same strategy was used to prepare plotted scaffolds on the basis of mineralized collagen, a nanocomposite developed for bone tissue engineering. The suitability of the plotted scaffolds for soft tissue engineering (collagen) as well as bone tissue engineering (mineralized collagen) was evaluated in cell culture experiments with human mesenchymal stem cells. The cell-matrix constructs were cultivated in the presence of adipogenic and osteogenic supplements, respectively. Our data revealed the cytocompatibility of the developed scaffolds since the cells were able to adhere, proliferate and differentiate towards the adipogenic and osteogenic lineage, respectively, as proven by microscopical, biochemical and molecular biological analyses.
Aortic valve (AoV) stenosis is a widely diffused pathology, resulting in native valve substitution with artificial or bio-prosthetic valves. In this work, we used a high throughput material screening approach to identify novel materials that may be used to grow aortic valve interstitial cells (aVICs) with physiological phenotype. Such materials would represent an optimal scaffold for delivery of tissue engineered heart valve (TEHV) novel prostheses. Polymer arrays were realized by contact printing (QArraymini with aQu solid pins from K2785, Genetix, UK) of a 300 polyacrylates (PA) library onto glass slides. Each polymer was printed in quadruplicate on the array. Primary human aVICs, obtained by enzymatic dissociation of pathologic AoV leaflets, were expanded for two passages on plastic before seeding and culture onto the arrays (3E+5 cell/array) for 72 hours (three replica experiments with aVICs isolated from three donors). Fluorescence staining for phalloidin, vimentin, collagen I, αSMA and DAPI were performed followed by fluorescence image acquisition and ImageJ analysis. The screening identified eight polymers promoting aVICs adhesion (between 34±1 and 10±3 cells/spot) without affecting viability. Adhesion onto these substrates determined various degrees of cytoskeletal organization. Studies are currently ongoing to further investigate interactions with these substrates and aVICs, as a step toward derivation of fully engineered TEHVs.
Microfabrication of Digitally Specified, Spatially Heterogeneous, Engineered Tissue Architectures

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Engineered tissues remain challenging to create, especially if the goal is to spatially position multiple cell types in a heterogeneous pattern in three dimensions (3-D). Borrowing from microfabrication techniques used in the semiconductor industry, we developed a simple and inexpensive way to create 3-D tissue prototypes by patterning hydrogel photocrosslinking via a process that can trap extracellular matrix components, cells, and diffusible factors in defined 3-D shapes, without requiring specialized optics or robotics. We demonstrate the power of this process by engineering tissue building blocks in defined geometries, creating engineered tissues that encapsulate different cells (e.g., primary neurons, embryonic stem cells, human umbilical vein endothelial cells, and fibroblasts) at specified locations throughout a single 3-D tissue volume. We have shown that cellular composition and concentration in each building block can be controlled spatially. Engineered neural networks comprised a complex population of neural cells, including excitatory, inhibitory neurons as well as glial cells, appearing at comparable ratios to that in the native cortex. Our results also suggested that neuronal growth and neurite development are regulated by available 3-D volume. This advancement allows us to envision building millions of digitally specified 3-D tissue prototypes with predetermined biomaterials, molecules and cell types at complexity and throughput levels never attained before.
A Miniaturized 3D Cellular Microarray Platform for Bone Tissue Engineering

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There are over 200 million people suffering from bone disorders worldwide today; many of them are diagnosed with severe skeletal defects that require (3D) bone graft transplants. Cellular therapies that utilize renewable cell sources could meet this requirement and repair or replace the damaged tissue. Currently human mesenchymal stem cells (hMSCs) are among the most promising cell sources for bone regeneration. In the past few years, numerous attempts have been made to direct hMSCs fate inside 3D microenvironments for treatment of bone related diseases. However none of the existing approaches have been applied to fully embrace the combinatorial nature of the stem cell niche. Here we present a three-dimensional (3D) cell-culture microarray platform that enables a rapid and efficient screening of stem cell fate inside combinatorial microenvironments. The proposed platform was generated with a robotic DNA printer capable of spotting nanoliter droplets. The droplets contained a UV-crosslinkable gel solution mixed with hMSCs and various extracellular (ECM) proteins. In total, we studied the influence of 96 different miniaturized 3D niches on hMSC differentiation toward an early osteogenic fate by measuring alkaline phosphatase (ALP) expression. In addition, using highthroughput imaging technique, we found ECM combinations that are responsible for ALP upregulation. Furthermore, these cues retained their osteogenic properties in macroscale settings and stimulated bone-formation after 25 days in vitro culture. Our 3D cellular microarray platform is therefore a powerful tool for revealing suitable microenvironmental conditions for osteogenic differentiation in a cost-efficient manner.
Tissue engineering using scaffolds is one of recent attempt to reconstruct the damaged or diseased tissues. However, the fabricated scaffolds with biomaterials could cause immunogenic reactions and complications during their degradation after implantation. Scaffold-free 3D tissue printing is a new and powerful approach in tissue engineering. In this research, optimized 3D design and path-planning for 3D tissue printing using cell aggregates as building blocks (bioink) is presented. We developed a novel computer-aided design and 3D printing technique to optimize bioprinting of scaffold-free tissue constructs. Computer-generated scripts directly from 3D computer-aided designs were used to control the 3D-bioprinter. Bioink made of cells were printed using bio-inert, thermo-responsive hydrogels as support structure. We optimized 3D path planning and bioprinting techniques essential for engineered 3D tissues and functional units.

Computer-aided biomodeling and path planning for 3D Bioprinting

Hybrid bio-printing with cells and support hydrogel
A major concern in tissue engineering (TE) is the limited possibilities for vascularizing artificial tissues. 3D bioprinting allows depositing biomaterials and cells simultaneously in specific patterns. Various tissues can be printed next to each other. It is especially suitable for more complex units where patterns are impossible to create by hand and also for repeated structures within the construct. Aim of this project was to simultaneously build different vessel structures within 3D bone tissue layer-by-layer. An alginate-gelatin hydrogel was enhanced with 8% hydroxyapatite (HA) in the bulk material; e.g. the bone phase, while no HA was included in the vessel phase. Inclusion of HA enabled visibility in micro-computed tomography (mCT), thus the bone phase could be distinguished from the vessel phase for the assessment of the printed structures in 3D. Constructs were printed by extrusion of continuous filaments. hMSCs were used for cell viability assessment; cells were mixed into hydrogel precursor before printing and analyzed after 3 days. Different vascular systems were realized with 3D bioprinting and compared by means of mCT. Smallest vessel diameter with the vessel oriented horizontally to the printing plane was $0.3 \pm 0.1 \text{ mm}^2$ and $6.8 \pm 0.9 \text{ mm}^2$ in vertical orientation. Cell-compatibility of material and printing process was proved detecting viable cells 3 days after printing. We developed a cell-compatible hydrogel suitable for generating vascular systems in 3D tissues. Vessels can be generated as lumen and used for medium perfusion or filled with cells aiming for cellular self-assembly process.

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The Effect of Porosity on Cell Ingrowth in 3D Laser-fabricated Biodegradable Scaffolds for Bone Regeneration

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Bone is the second most common transplantation tissue after blood. While the use of bone grafts remains the optimum choice, the problems associated with them has made the use of synthetic implants ever more popular. Over the last decade, there has been a lot research into the development of engineered new bone, to replace damaged tissue. An important part of this research effort has gone into the development of three-dimensional porous scaffolds, to support and guide the new cells. Here, we describe our research into the fabrication and evaluation as bone scaffolds of 3D biodegradable structures made using Direct fs Laser Writing (DLW). The material we use is a photostructurable polylactide-based material (PLA) synthesized for this purpose. We test its degradation \textit{in vitro} in PBS and we show that the material looses one third of its weight after six weeks, therefore allowing the slow release of an implanted scaffold. We demonstrate the fabrication of artificial scaffolds with precisely controlled geometries and different pore sizes and we test them for up to eight weeks using the mouse pre-osteoblastic cell line MC3T3-E1. Our results show good cell adhesion and a preference to scaffolds with 86% porosity, compare to other porosities studied. Our study shows that DLW is a suitable technique for the fabrication of 3D biodegradable scaffolds for bone repair and other tissue engineering applications.

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Porous non-biodegradable polyethylene implants for treatment microtia have suboptimal material properties. Additive manufacturing opens unique opportunities for rational design and fabrication of patient specific biocompatible auricular implant with optimal material properties maintainable after implantation. The patient specific porous polyurethane auricular implant have been designed using laser scanner, finite element analysis, CAD software and fabricated using fused deposition modeling. The material properties of human cadaveric auricular cartilage and fabricated implants have been tested using three points flexure method. The biocompatibility and maintainability of fabricated auricular implant size and geometry have been tested in nude rats. The patient specific porous polyurethane auricular implants have been fabricated using fused deposition modeling. The optimal material properties comparable with native cadaveric human auricular cartilage have been achieved using rational design approach based on finite element analysis. The original shape and geometry of implant have been maintained up to 3 months after implantation with optimal level of biocompatibility. The patient-specific biocompatible porous polyurethane auricular implant with optimal material properties sustainable after implantation could be fabricated using fused deposition modeling. The hybrid approach with simultaneous deposition of hydrogel filaments containing chondrocytes between polyurethane filaments will enable bioprinting of patient specific hybrid tissue engineered auricular implant with optimal and maintainable material properties.
Native tissues are composed of three dimensional (3D) units characterized by a microscale spatial organization defining cell-cell and cell-extracellular matrix interactions. The ability to replicate this 3D architecture is fundamental to effectively engineer biomimetic constructs. With this aim, we developed a photo-mold patterning (PMP) protocol to obtain cell-laden microgels with user-defined geometrical features. The PMP enables for rapid and controlled cell embedding within hydrogel micropatterns, by photopolymerization in PDMS stamps used as geometrical constraints. Synthetic and naturally-derived prepolymers (Polyethylene Glycol Diacrylate and Gelatin Methacrylate) were crosslinked in the presence of a biocompatible near-ultraviolet (UVA) photoinitiator (VA-086, 1.5% w/v), activated through a low cost LED light source. The protocol was validated both on a continuous cell line (Human Umbilical Vein Endothelial Cells expressing Green Fluorescent Protein, HUVEC GFP) and on primary human Bone Marrow Stromal Cells (BMSC). PMP allowed to achieve micropatterns narrowly replicating 250 µm wide and 80 µm high features, characterized by a 3D uniform distribution of cells. Morphology evaluations during a period of 7 days in vitro (DIV) showed a tendency of cells to spread, migrate and reorganize within the microgels, either forming network-like structures (HUVEC GFP) or aligning along the main axis of the pattern (BMSC). Cell metabolic activity also increased from DIV 1 to DIV 7, demonstrating the PMP potentiality to form biocompatible 3D microenvironments. PMP can thus be considered a promising and cost effective tool for designing spatially accurate in vitro models and, in perspective, functional constructs.
Microfluidic Transwell Platform to Recreate Physiological Conditions and Epithelial Structure of Renal Proximal Tubule

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Excreted urine results from a highly regulated process, in which, initial blood filtration passes through several reabsorption processes to recover useful elements and discard toxins and metabolic residues. Study renal epithelium in “physiological like” conditions is hard to achieve by \textit{in vitro} classical methods and finally requires the use of animal experimentation even in early steps of research. In this work we present a microfluidic “transwell” device for the creation of a biomimetic cell culture platform for renal proximal tubule cells. Devices have been designed to obtain two microchambers separated by a permeable membrane. Each microchamber has independent microfluidic channels, allowing the use of different liquids passing through the microchambers at the same time, recreating blood and urine in kidney (Figure 1 a)). First microfluidic chips have been successfully fabricated by SU-8 technology \cite{1}, where shear stress values near to physiological proximal tube levels can be obtained (Figure 1 b)). Further experimental work includes cell culture validation and functionality assays, results will be presented in the congress. The use of the device presented in this work could therefore decrease and delay the use of animal experimentation, which would have a big ethical and economic impact with respect to nowadays technologies.

![Figure 1. (a) Cross section scheme of Su-8 based microfluidic transwell. (b) Image of fabricated microfluidic transwell.](image)

Hybrid Scaffold Fabrication Using Dual Head Scaffold Plotting System


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Scaffold has been widely studied in the field of bone and cartilage tissue regeneration since it serves as a three-dimensional (3D) supporting structure for tissue formation. Hydrogels are one of the good candidates for the fabrication of scaffold. But the hydrogel scaffold needs good mechanical properties to keep the shape under the mechanical loading during the implantation. In this study, we designed and fabricated a novel two head scaffold plotting system (2H_SPS) which is based on the solid freeform fabrication (SFF) technology. The 2H_SPS can fabricate the load-bearing polymer matrix and cell-laden hydrogel matrix in a single 3D hybrid scaffold. We used polycaprolactone (PCL) to increase the mechanical strength of the scaffold and alginate to encapsulate condrocyte in the scaffold. The experiment results show that the 2H_SPS can fabricate hybrid scaffolds with interconnected porous structures with different pore size and porosity. The confocal images of the hybrid scaffold show that the chondrocytes are homogeneously distributed among the alginate matrix. The mechanical properties, proliferation and differentiation of the cells are also compared with the general hydrogel scaffolds. To measure the degree of cell proliferation, WST-1 cell proliferation assay kit (Takara, Japan) was used. And the total amounts of glycosaminoglycans (GAG) per scaffold were measured to compare the cell differentiation.
The aim of the study was to investigate the influence of internal architecture of 3D printed scaffolds on their mechanical and biological properties. Polycaprolactone scaffolds with six different geometries produced by Fused Deposition Modeling (FDM) were tested in this study. The 3D samples were manufactured with different lay-down pattern of the fibers by varying the layer deposition: 0°/15°/30°; 0°/30°/60°; 0°/45°/90°; 0°/60°/120°; 0°/75°/150° and 0°/90°/180° (Fig.1).

3D structure and internal architecture were investigated by scanning electron microscopy (HITACHI SU8000) and microtomographical analysis (SkyScan 1172). Compression tests of the samples were carried out with a Zwick tester (Zwick Z005) at a cross-head speed of 1 mm/min up to 50% of compressive strain. After γ-sterilisation, stem cells were seeded in the scaffolds (0.5 x 10⁶/scaffold), expanded for 7 days in proliferation media, and cultured for 1 day and 28 days in basic, mineralization and chondrogenic media. Cell growth and differentiation toward the chondrogenic and osteogenic lineage were evaluated by DNA, ALP and GAG assays. The obtained scaffolds had structures with high porosity (50-60%) and interconnected pores ranging from 380 to 400 μm. Changing the angle deposition affected significantly the mechanical properties of the scaffolds. The highest Young’s Modulus was observed for the samples with 0°/60°/120° and 0°/90°/180° lay-down pattern. Cellular studies also showed an influence of the internal architecture on cell adhesion and proliferation within the construct. Summarizing, by using FDM technology it is possible to fabricate scaffold with controllable geometrical parameters, good mechanical properties and biocompatibility.

Keywords: Scaffolds, internal architecture, polycaprolactone, human mesenchyme stem cells, mechanical properties
Preparation of Biomimetic, Digital Phalanx Scaffold for Tissue Engineering Using Micro-CT and Three Dimensional Printer

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To prepare biomimetic, intelligent scaffold for tissue engineering of thumb distal phalanx using gathered three-dimensional (3D) data. Distal phalanges from fresh adult cadaver thumbs were subjected to micro-CT scanning. The DICOM data from micro-CT scans were processed using Mimics software to form the macro and micro biomimetic 3D printing STL file. A 3D printer was then used to print out a scaffold of a phalanx. The data acquired by Micro-CT scanning can be rapidly prototyped after being edited with Mimics and directly used by 3D printing system to construct a biomimetic, intelligent scaffold. Biomimetic, intelligent scaffold based on micro-CT and 3D printing has clinical significance in reconstruction of destructed thumbs.
This work presents the design and fabrication of SU-8 based microfluidic chips for cell culture applications. The novelty of the system relies on the integration of 3D scaffolds, which allow the study of 3D cell growing under microfluidic control conditions. Due to the SU-8 transparency and that it is a polymer, these chips are compatible with optical inspection and NMR. Furthermore, SU-8 based cell culture microfluidic devices hold the potential to allow the constructions of advanced biomimetic systems. Its capability to build 3D microfluidic networks and the availability of monolithic integration strategies for microsensors and microactuators make SU-8 technology a promising route for next generation of cell culture microfluidic devices. We have designed a microfluidic chip based on SU-8 technology. The design includes a culture chamber where the scaffold is located and lateral microchannels. The aim of these microchannels is the correct perfusion of media through the culture chamber to keep the cultured cell with the proper level of nutrients and oxygen. First devices have been successfully fabricated, obtaining a chip with a culture chamber of 300 μm height and the correct insertion of the scaffold on it. The scaffold material is completely compatible with the different microfluidic fabrication steps that should support temperatures of 90°C with no degradation. Biological assays and the corresponding results will be also presented.
Multi-functional Heterogeneous Tissue Scaffolds

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A novel tissue scaffold design technique has been proposed with controllable porous architecture design suitable for bio-additive manufacturing processes. The proposed layer-based design uses a bi-layer pattern of radial and spiral layer consecutively to generate functionally gradient porosity. The proposed approach constructs the medial region from the medial axis of each corresponding layer, which is represents the geometric internal feature or the spine. The radial layers of the scaffold are then generated by connecting the boundaries of the medial region and the layer’s outer contour. Gradient porosity is changed between the medial region and the layer’s outer contour. The combination of consecutive layers generates the pore cells with desired pore sizes. To ensure the fabrication of the designed scaffolds, the generated contours are optimized for a continuous, interconnected, and smooth deposition path-planning. A continuous zig-zag pattern deposition path crossing through the medial region is used for the initial layer and a biarc fitted iso-porosity curve is generated for the consecutive layer. The proposed methodologies is used to generate the structure with gradient (linear or non-linear), variational or constant porosity that can provide localized control of variational porosity along the scaffold architecture. The designed porous structures can be fabricated using bio-additive fabrication processes.

Generated
Osteogenic Differentiation of Human Adipose-Derived Stem Cells on Polycaprolactone/Tricalcium Phosphate Composite Scaffolds

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We investigated the feasibility of ε-polycaprolactone/tricalcium phosphate composite scaffolds (PCL/TCP) to support osteogenesis of human adipose derived stem cells (ASC). Cylindrical, porous scaffolds were manufactured with Bioscaffolder® machine (SYSENG, Germany) by plotting the melted composite with a 330µm dispensing needle layer by layer, with lay-down pattern of the fibers: 00/600/1200 with shifted layers. ASC cells were pre-induced by culture with vitamin C containing medium and seeded onto the scaffolds at passage 2. After 24 hours, mineralization medium was applied and the cells were cultured for 14 or 42 days. The cells proliferated efficiently on scaffolds which was shown by a 10-fold increase in Alamar Blue assay readout after 14 days. Live/dead fluorescent staining revealed that cells covered the whole surface of scaffolds at day 14 and filled scaffold pores at day 42. SEM analysis showed the onset of extracellular matrix deposition at day 14, which increased at day 42. This observation was further confirmed by fluorescent staining of hydroxyapatite. At day 14, a number of mineralization nodules was visualized while at day 42 the scaffold was covered by a layer of hydroxyapatite. Furthermore, up-regulation of Runx2 and collagen I genes expression was observed after 14 days of culture. Finally, cells detached from the scaffolds after 14 days produced osteonectin protein which was evidenced by FACS analysis. These results indicate that human ASC cells produce mineralized extracellular matrix and acquire an osteoblast-like phenotype on PCL/TCP scaffolds. Therefore those bioconstructs could be potentially used for bone reconstruction therapies. Grant no POIG.01.01.02-00-022/09.
Nanostructured scaffolds have a great potential in tissue engineering and regenerative medicine. Complex extraordinary mechanical, chemical and biological criterions are demanded. Stable three dimensional macroscopic shapes consist of highly porous defect free nanofibers made of biocompatible, biodegradable and nontoxic material is still not achieved. Therefore there is a great interest to develop both not only such scaffolds but production methods and procedures well-designed for this purpose as well. New desktop laboratory apparatus have been developed for deposition of nanomaterials dedicated to medical applications. The apparatus integrates different methods to enable preparation of nanostructured scaffolds according to researching demands. These methods are Electrospinning, Electrospraying, Electroblowing and Solution spinning. Almost twenty different materials (including hyaluronic acid and its derivates) have been processes so far. Precisely aligned nanofibers with anisotropic properties have been collected by advanced Electrospinning. Small balls structures have been prepared in the Electrospraying mode. Morphological properties could be well controlled by Electroblowing process parameters, for example reduction of fiber diameter down to 50 % is obtained. The device is designed to be run in a clean room (variety of accessories are able to be sterilized). Stable accurate processes lead to preparation of material with high repeatable and reproducible properties. Thanks to safety components, easy handling, intuitive device control and other benefits the apparatus significantly contribute to accelerate research progress in the medical application field.
The concept of using magnetic responsive scaffolds provides new tools to attract stem cells or biomolecules, trigger differentiation pathways and even control constructs via magnetic manipulation after \textit{in vivo} implantation. This study focus on the development of 3D magnetic scaffolds using a 3D printing fabrication method, through the incorporation of magnetic nanoparticles (MNPs). Commercial iron oxide (Fe$_3$O$_4$) nanoparticles (Micromod) were dispersed within a biodegradable blend of starch and polycaprolactone (SPCL/Fe$_3$O$_4$) (1:0.018 ratio, w/w). 3D fiber scaffolds were printed (Envisiontec) into multiple hierarchical layers building different geometric structures. The fiber orientation was established as 0° or 0°/90° between successive layers. Magnetic responsive scaffolds were successfully developed in a simple and versatile technique. Various architectures were obtained without affecting the magnetizing capacity of the scaffolds, which were verified by attraction to a magnet. The microstructure of the fabricated scaffolds was characterized by SEM and μCT. EDS mapping show uniform and homogeneous distribution of the MNPs in the fiber network both at surface and within the fibers of the scaffolds. Ongoing cell studies are expected to elucidate the scaffolds potential in tailoring the behavior of human adipose tissue- and amniotic fluid-derived stem cells so as to enhance functionality of bioengineered constructs. Promising magnetic approaches on scaffold complexity as well as cell development are envisioned for tissues susceptible of magnetic stimulation, as bone or tendon. The newly fabricated scaffolds with magnetic features have the potential to impact the field of skeletal tissues regeneration.
Towards 3D Artificial Cardiac Valve Printing: Design And Physical Specifications

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Heart valve diseases are causing at least 40,000 deaths per year worldwide. The use of tissue engineering to create lifelong and easily implantable artificial heart valves have been proposed as a promising clinical solution. Artificial valves would solve thrombogenicity, structural deterioration and risk of infection overcoming the majority of problems related with traditional prosthesis.

Printing a heart valve requires detailed knowledge of biological valve characteristics as well as its biomechanics. Our aim was to improve valve 3D printing procedures, using the available control systems. Accurate control of printing process involves the appropriate definition of the operating parameters for each type of cells and scaffold materials. Actual printers have several limitations, which should be studied in order to know the real possibilities for printing cells. A literature search combined with iterative simulations were carried out using viability measurements and results from previous tissue engineering experiments with human adipose stem cells (hASC). We payed special attention in monitoring results about temperature variations, pressures and other parameters that may be key points for a properly printing phase. This also helped to identify the most significant and relevant factors in the printing procedure that guarantee the viability and health of the cells.

We can adjust printer requirements to maintain cell viability, proliferation and differentiation events correlates well according with our hypothesis. Therefore, rapid prototyping could provide important insights into the design parameters and demonstrate the principal feasibility of printing a heart valve using a cell printer system.
Electrospinning polymers into nanofibrous scaffolds is a technique that has been used in practically all areas of tissue engineering (TE) applications. Limitations in electrospinning include its inability to control where the polymer jets land, and the production of small pore size preventing cells from penetrating the scaffold. Electrospraying of living cells to a targeted area is another proven jetting methodology. These jets use needles to form nearly mono-dispersed droplets of cells in the micro range. The aim of this project is to use a novel electrospray technology developed at National University of Ireland Maynooth (NUIM) for tissue engineering and regenerative medicine applications. 3D extracellular matrix components are sprayed onto a specific area to generate micrometer pores for cells to fully penetrate the matrix. Bone marrow-derived mesenchymal stem cells (BMSC) were chosen as these are non-hematopoietic progenitor cells that can differentiate into ectodermal cells (neural), mesenchymal cells (adipose, chondrocytes) and endodermal cells (hepatocytes). Mouse BMSC are sprayed in 2D culture and onto 3D polyethylene scaffolds with a working voltage of ~3.5kV @ 3µL/min. These cells adhere and proliferate successfully. Current work involves electrospraying the 3D extracellular matrix components after which the BMSC will be sprayed on top. These cells will then be tested for cell infiltration and distribution into 3D matrices, and their ability to continually differentiate into multiple cell types will be determined.
Keratins belong to the family of fibrous structural proteins and are the basic building blocks of feathers, hair and wool and the key structural materials of the outer layer of human skin and nails. Due to their unique chemical composition, biological activity and biocompatibility, keratins biomaterial is an appealing choice for therapeutic development. Firstly, extracted keratin proteins have the ability to self-assemble and polymerize into porous, fibrous scaffolds. Secondly, keratin biomaterials derived from wool and human hair have been shown to retain active cell binding motifs, such as LDV (Leu-Asp-Val) and RGD (Arg-Gly-Asp) binding sites, which are capable of supporting cellular adhesion and motility. Exploiting these properties, in the present work, keratins were used to functionalize several substrates. Solution of keratin hydrolysates at different percentages (from 2.5 to 30 %) were printed using a modified 3D thermal inkjet printer, Penelope, on glass slides, PMMA films, alginate and gelatin hydrogels. Based on HP technology, Penelope is able to print several water-based solutions in a temperature controlled environment and with the possibility to print 3D object in with a layer-by-layer approach. Preliminary results with C2C12 mouse myoblast cell line demonstrated the feasibility of the chosen functionalization method.
The liver plays a central role in human drug interactions and is also the most common target for drug-induced toxicity, resulting in costly, late stage drug failures. In this project, we derive inspiration from the building blocks of the liver, the liver sinusoid and the acinus structure, to build a realistic microfluidic liver platform to accelerate drug testing and toxicology studies. The design consists of a microfabricated platform with parallel cords (microfabricated grooves) separated by thin walls. These cords house a three dimensional analog of the liver sinusoid populated with human liver cells. Using a step-by-step approach, we introduce primary hepatocytes into these cords, followed by the non-parenchymal cells (NPCs), including endothelial cells, stellate cells and Kupffer cells. These NPCs not only support optimal hepatocyte function but also directly contribute to drug metabolism and induced toxicological challenges. Using strategies to mimic in vivo flow characteristics, including nutrient and oxygen zonation, we aim to deliver a platform that closely mimics the in vivo liver and therefore produces data with improved predictive capabilities.
Laser Assisted Bioprinting for Mesenchymal Stem Cells

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In conventional tissue engineering (top-down) methods, there are limitation factors and challenges. These are cell localization arrangement, diffusion problems, organizing, taking long time of construction. Bioprinting abrogate all of these problems. Cells can be printed where you want. Laser assisted bioprinting technology support that cells can be localized into biomaterial as three dimensional structures in short time and organizing cells can be controlled with high sensitivity. Biological materials are configured layer by layer from bottom-up by using computer aided design. Tissue fusion or directed tissue self-assembly is a fundamental principle of bioprinting. The aim of this technology is becoming to print new organs. Laser Assisted Bioprinting includes three main steps. These are 1. Preprocessing (CAD, blueprint), 2. Processing (bioink, biopaper, bioprinting, solidification), 3. Postprocessing (tissue fusion, tissue maturation). This study is highly related with processing part. Bioink is a viscose solution has cells (MSCs, for this study) are printed. Biopaper is kind of biomaterial (collagen or matrigel, for this study). Exact bioprinting part has some parameters about laser kind, wavelength, power, frequency, speed and orientation. All of these parameters and properties are important to obtain printed alive cells with high throughput. In the process, laser induces to bioink and an inkjet is generated from there. After that, one droplet includes cells are placed into the biomaterial. Laser acts in accordance with blueprint until it is completed. Optimum parameters and other conditions for printing mesenchymals stem cells were optimize in this study. After printing process, live-dead viability cell assay was realized and alive printed cells could be seen brightly and in good morphology. This study shows, bioprinting is more effective, clear and easier than other tissue engineering methods.
Malignant cells show morphological, genetic and behavioral differences compared to the tissues they originate from. Osteosarcoma is a malignant mesenchymal neoplasm and most common primary malignant tumor of bone. Recent studies show that scaffold geometry and complex mechanical parameters of polymeric surfaces can induce certain biochemical responses in the cell which are defined by mechanosensing and mechanotransduction. These biochemical responses then trigger certain signaling pathways resulting in transcriptional changes, deformations and differentiation of the cell. PDMS templates were produced over silicon wafers bearing areas of square micropillars with 2-16 μm sides and varying gaps, and poly(lactic acid-co-glycolic acid) (PLGA) films were produced by solvent casting on these secondary templates. Saos-2 cells are human osteosarcoma cells and are used in this study as an in vitro malignancy model. Saos-2 cells were seeded on the films and they were evaluated via fluorescence and scanning electron microscopy after 48 hours. Saos-2 cells showed a range of responses from nucleus deformations to cell cytoskeleton alignment in relation to the dimensions of the pillars and the gaps. This test system may be used to distinguish malignant cells from healthy counterparts.
Rapid manufacturing (RM) is a process allowing not only the establishment of real 3D models as prototypes but also the fabrication of 3D objects, which is an absolutely novel application. Using appropriate rapid manufacturing processes, i.e. 3D printing for selective laser sintering, custom-made human heart valve scaffolds are to be fabricated for further seeding with vascular cells from human umbilical cords. Different resorbable polymers were analyzed for their suitability as potential scaffold materials. The optimal particle size of resorbable polymers for 3D-printing techniques was evaluated. All primary materials are available only as fibrous structures and granules which are not suitable for 3D printing due to the large particle size. Heating to specific temperatures to melt the structures and special grinding are necessary to generate a powder-like structure. Scaffold samples were produced using a contactless stripe-light 3D scanner and were analyzed for their cell-seeding capabilities. Different resorbable polymeric granules and powders based on polyglycolic acid (PGA) and polylactic acid (PLA) were successfully heated and ground and used to fabricate heart valve scaffolds by the rapid manufacturing process. Human vascular cells attached to the scaffold surface without migration into the inner structure of the polymeric samples. Since appropriate powder-like resorbable polymeric materials for 3D printing of heart valve scaffolds are not available on the market, special processes such as heating and grinding had to be evaluated. For future experiments porous polymeric scaffolds have to be generated using a 3D printer for selective laser sintering.

Keywords: rapid manufacturing, heart valves, resorbable scaffolds, tissue engineering
SU-8 Based Microfluidic Devices for Three Dimensional Cell Culture

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Extracellular matrix provides cells of a three dimensional (3D) environment. Such mechanical structure allows both, cell migration and nutrient and oxygen diffusion, through it. Furthermore, cellular communication is, commonly, mediated by biochemical gradients within this extracellular matrix. As a consequence, researchers have used hydrogels to recreate this physiologic 3D environment, since they allow cell growth and migration. More recently, hydrogels have been integrated within microfluidic devices, mostly based on PDMS technology. It definitely meant a clear step forward, as it allowed the use of microfluidics to control the hydrogel environment. However, PDMS exhibit some disadvantages, like molecule adsorption, deformability or uncontrolled gas exchange. In this work, we present SU-8 based microfluidic device for three dimensional cell culture systems. The final device was designed to provide a central culture chamber and two lateral microchannels. The device was fabricated using photolithography technology and SU-8 as structural material. Such fabrication strategy provides mechanical stability, avoiding any gas exchange through their walls. Once fabricated, a collagen gel, with cells embedded within it, was located at the central chamber by manual perfusion. The properties of the device allowed an easy gel confinement at the culture chamber, avoiding strict device design requirements or any treatment. Once gel polymerization took place, nutrients were delivered by diffusion from medium flow through lateral microchannels. Moreover, biochemical gradients could be established across the gel. The results obtained while applying an oxygen gradient within the device will be presented at the conference.
Cell-dispensing Process for Fabricating Alginate Scaffold Embedded with Preosteoblasts

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To fabricate three-dimensional (3D) cell-laden alginate scaffolds, a modified dispensing method is presented. To evaluate the process, preosteoblasts (MC3T3-E1) mixed with an alginate were fabricated into a 3D matrix. The fabricated cell-laden structure was highly porous and had a uniformly designed pore size and shape. We compared two different dispensing nozzle sizes by observing the cell distribution in the struts and determining the cell viability within the scaffolds. The scaffold embedded cells were 85% viable compared to the initial cell viability, and the cell distribution was homogeneous. The 3D pore structure of the alginate scaffolds consisted of 100% interconnected pores with channels extending uninterrupted from top and bottom. Although we initially designed the pore as a complete rectangle, the final product had a slightly rounded shape due to some dissolution of the alginate struts at the contact region of each strut. To determine the effect of the nozzle size on cell viability, two different nozzle sizes (outer diameter = 310 and 610 μm) were used. The cells were simultaneously dispensed with the alginate solution (3.5 wt%) to measure cell viability throughout this process. The cells were homogenously distributed throughout the alginate struts. The viability of the dispensing process was determined to be 85 ± 3% for the large nozzle size [610 μm], and 84 ± 5% for small nozzle size [310 μm]. The values were high, despite the fact that the cells were extruded using a high pneumatic pressure in the dispensing barrel (40 and 210 kPa). In addition, the difference between the viability for the two different nozzle sizes was negligible. To characterise the spatial distribution of the cells, confocal microscopy which indicated that the cells were homogenous and well distributed in the inner alginate struts for both scaffolds, was performed. The fluorescence and confocal microscopy data indicate that the preosteoblasts were homogenously distributed in the alginate scaffold and the cell viability was reasonable for tissue regeneration.
A small population of human corneal stromal stem cells (hCSSCs) have been identified in the corneal stroma which display properties similar to mesenchymal stem cells (Du et al., 2005). These cells demonstrate the ability to reproduce an organized matrix in vitro and therefore have great potential for stromal tissue bioengineering and for the development of cell-based therapies for corneal blindness. Previously, it has been shown that these cells can be harvested from donor tissue that is less than 6 days old and stored in Optisol storage medium. The aim of this study was to investigate the properties of hCSSCs to determine whether they retain their phenotype when cultured in vitro from long storage organ-cultured corneas. Donor corneas that had been stored either in organ culture medium (OC) up to 4 weeks (n=3) or in Optisol medium (OS) up to 6 days (n=3) were used for isolation of hCSSCs following the method described by Du et al., 2005 and maintained in culture up to passage four. The phenotypes of cells from the two cultures were examined with light microscopy and immunocytochemistry (PAX6, CD73 and CD90). PAX6 protein expression was further confirmed with immunoblot analysis. A comparison of both cultures revealed no differences in the morphology of hCSSCs between OC corneas and OS corneas. Immunocytochemistry revealed hCSSCs from both OC and OS corneas, maintained positive staining for PAX6. Positive expression of mesenchymal stem cell markers CD73 and CD90 were also observed in cells from both cultures. Protein expression of PAX6 in cells from both tissue types was demonstrated by immunoblotting. This study demonstrates that hCSSCs exhibit a robust survival capacity by retaining their phenotype following isolation from long storage, organ-cultured corneas. Surprisingly, no significant difference was observed in their morphology, expression of PAX6, CD73 or CD90. This level of robustness to such storage conditions allows the use of hCSSCs from organ cultured corneas which are not only more widely available for research but also often discarded for retrieval of other cell types such as corneal epithelial and endothelial cells which require high tissue quality for their preservation.
Development of Rich in Extracellular Matrix Human Corneal Fibroblast Cell Sheets

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The clinical translation of scaffold-free tissue constructs (self-assembly approach) has been limited by the prolonged culture time. For example, culturing corneal fibroblasts (HCFs) need 35-84 days, still results in the production of tissue containing less extracellular matrix (ECM) and therefore is thinner than native corneal tissue. Recent studies have shown that macromolecular crowding (MMC), the addition of macromolecules to culture media, not only enhances deposition of extracellular matrix (ECM), but also facilitates cell phenotype maintenance. Herein, we assess the influence of MMC on ECM deposition of HCFs culture using 100μg/ml dextran sulphate (DxS); 37.5mg/ml Ficoll™ (Fc) 70 and 25mg/ml (Fc) 400; and 50μg/ml Carrageenan (CR). ECM deposition was analysed by SDS-PAGE, immunocytochemistry (ICC) and proteomic analysis. Cell phenotype maintenance was assessed using RT-PCR. Rich in ECM cell-sheets were successfully detached using a thermo sensitive polymer; NIPAAM and n-tert butyl acrylamide. Densitometric analysis of SDS-PAGE and complimentary ICC analysis demonstrated significant increase in collagen I deposition (p<0.05) up to six days. Proteomic analysis confirmed the increase in ECM/total proteins deposition. Gene analysis indicated no alteration in cell phenotype. The novel thermoresponsive polymer enabled the detachment of transparent and ECM-rich HCFs cell sheet in 30 minutes at 200C. This novel technology offers a promising avenue for the generation of corneal tissue equivalents with a well developed corneal stroma. The authors would like to thank the College of Engineering and Informatics, NUI, Galway, SFI (Grant-07/IN1/B031, 09/ RFP/ENM2483) and SFI- ETS Walton award (MR) for financial support.
Limbal epithelial stem cell deficiency causes loss of corneal epithelial integrity and impaired vision. We have developed a plastic compressed collagen construct (RAFT) with good optical and mechanical properties to carry limbal epithelial cells (LEC) to transplantation. To test LEC function on RAFT, re-epithelialisation (R-E) after injury was measured. LEC were isolated from human corneas (N=3), expanded, seeded onto 16mm diameter RAFT without limbal fibroblasts (LF) and cultured for 2 weeks to form confluent monolayers (mRAFT) (n=17). Others were cultured a further week at air/liquid interface to form stratified epithelia (sRAFT) (n=16). Heptanol-soaked paper discs were used to create central 5mm diameter wounds on RAFT. R-E was observed post-wounding (PW) using 10uM fluorescein diacetate (FdA), which is cleaved to a fluorescent product by viable LEC. RAFT were incubated with FdA for 2 mins and photos taken under blue light through yellow glass. Immunohistochemistry for putative stem cell marker p63alpha was performed after R-E. FdA staining revealed that LEC closed all wounds on mRAFT by day 8 PW. When sRAFT were wounded, 2/16 healed by day 7 PW but the remaining 14 failed to heal and FdA staining was sparse by day 14 PW. P63alpha expression was 3-fold greater on mRAFT cf. sRAFT: 22±9% cf. 7±5%. This suggests that mRAFT may be sufficient for transplant, thus reducing delivery time to patient. Inclusion of LF within RAFT may further enhance LEC function.
Fibroin Fiber for Artificial Corneal Stroma

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Because of the donor shortage, development of novel artificial cornea has a meaning to a person who suffering corneal blindness. At present, many kinds of artificial corneas have been developed. However, perfectly reliable material has not been established yet especially for corneal stromal site. Therefore, for developing the novel artificial corneal stroma is significant. Main components of corneal stroma are collagen layers and keratocytes. Each collagen layer in corneal stroma has highly aligned structure. Thus, we focused on aligned fiber mat for artificial corneal stroma. Silk fibroin is known as a natural material which has good biocompatibility. We could fabricate the aligned nanofiber non woven mat from aqueous fibroin solution using electrospinning method. However, as reported in last TERMIS world congress, our fibroin nanofiber non woven mat had critical weak points such as transparency and difficult handling. Therefore, we improved the transparency and handling of our material using gel - fiber hybrid system and fiber-stretching system. Using the gel - fiber hybrid system, we achieved to create a transparent material and handling was improved by stretching system. Details of in vitro and in vivo data of our novel artificial cornea will be discussed in the conference.
Corneal Stromal Cell Plasticity: In Vitro Regulation and Restoration of Keratocyte Cell Phenotype Through Cell-Cell Interactions In a 3D Model

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In vivo, epithelial cells are in close contact with keratocytes in the stromal layer; they are connected both anatomically and functionally, and it is these interactions that are vital to the maintenance of tissue homeostasis and transparency. Co-culture studies aim to recapture this cellular anatomy and functionality by bringing together two or more cell types within the same culture environment, enabling them to interact and communicate which can act as a very powerful in vitro tool. The influence of cellular interactions is of particular interest to tissue engineers because the tissue formation of one or all cell types can be regulated by simulating and stimulating the natural physiology and differentiation of cells. In order to more closely mimic the native niche environment three different co-culture methods have been examined; epithelial explant; transwell; the use of epithelial conditioned media and their effects on stromal cell function were studied. The different co-culture models help us to determine as to whether cell-cell interactions are due to direct cell contact or not, and as to whether the cells themselves have to be present to elicit a response. By more closely mimicking the in vivo cellular niche environment we are able to aid our understanding of corneal wound healing mechanisms and the importance of cell mediated interactions; thus enabling us to engineer corneal tissue with stromal cells that are in a healthy, native, uninjured (non-activated) state. The effects of the addition of transforming growth factor β-1 (TGF-β1) and wortmannin to the culture media was also investigated to examine cell plasticity within the corneal model and whether this can be blocked.
Tissue Engineered Drug Delivery System for Ocular Surface Treatment

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Current treatment for corneal disorders typically require frequent and prolonged topical administration of medication due to poor drug penetration and high clearance rate. This can be overcome by prolonging the drug contact time at the cornea. In addition to direct topical drug application, amniotic membrane (AM) patches onto the cornea are used for adjuvant management of eye injuries or infections, but the AM is biochemically variable, hard to handle and carries the risk of disease transmission. The aim here was to test the use of dense plastically compressed (PC) collagen, loaded with drug-encapsulated nanoparticles (NPs) as a controlled drug delivery for corneal infections. Collagen gels containing one of 2 types of NPs were subjected to PC to rapidly remove most of the fluid to form dense NP-loaded collagen sheets. Entrapment efficiency and spatial distribution of hyaluronan-NP was determined using FITC-tagged particles. This showed a trapping efficiency of 18.5-31.1% depending on configuration. Similarly, the entrapment efficiency of chitosan (quaternary ammonium palmitoyl glycol chitosan (GCPQ)) nanoparticles loaded with nile red (NR) was determined to be 18.8-36.2%. GCPQ particles carrying a cargo of the antibiotic, Sparfloxacin, were used to test effective drug release by this system. Sustained release of active Sparfloxacin was demonstrated to be capable of killing target microorganisms. Thus this mass-produced, reproducible nanoparticulate-scaffold system allows for the controlled release of drug directly at the target site, with the capability of use with alternative drugs and other applications.
Efficient biological glue can replace surgical sutures. Enzyme transglutaminase-2 (TG2) stabilizes connective tissue by covalent crosslinks. We hypothesize that TG2 based biological glue will covalently bond tissues to replace sutures on corneal wounds. Our aim is to mechanically test the TG2 glue strength for corneal repair. The TG2 glue is a combination of the recombinant human TG2, dendrimer enzyme substrates and calcium buffer. In the test these 3 components mixed causes the glue to set and in the control EGTA replaces calcium which abolishes the enzyme activity. The glue strength in cornea was studied in cadaveric porcine corneal flaps by delivering the glue to the interface, closure, incubation and then mechanical testing with instron. Ability of the glue as a sealant to prevent leakage of intraocular fluid across the incisions glued was also tested by applying a measured increasing intraocular pressure till the glue failed. The mechanism of the glue failure was studied by microscopic visualization of cryosections of the fused tissue stretched on transparent membrane. Gaping of the fusion line was recorded at different strain points. In all the tests fibrin glue and the sutures were used as benchmarks. The strength of the TG2 glue was found to be significantly higher than the benchmark fibrin glue. Even though the strength of the sutures were superior to the glues the values of the TG2 glue strength was comparable to the sutures as well as acted as a good sealant preventing leakage across the corneal incisions. Microscopy of the glue failure indicated a superior cohesive and adhesive strength of the TG2 glue over the fibrin glue. Cornea is an ideal target for TG2 glue based repair.
Surgical Procedure for Sub-retinal Transplantation in Age-Related Macular Degeneration (AMD)

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AMD is characterised by deterioration of retinal pigment epithelial (RPE) cells and their underlying Bruch's membrane (BM). Replacing these cells with a tissue engineered construct (TEC) of a cultured monolayer of cells on an underlying artificial membrane could help restore vision. This study investigated subretinal transplantation of TECs of various sizes, shapes and encapsulation in an ex vivo porcine model following vitrectomy. aRPE-19 cells were cultured on 20μm thick polyurethane (PU) membranes until fully confluent. The membranes were 6mm discs or 3mm x 5mm strips. Five of each shape were precut prior to cell culture and the same amount were cut after cell culture using a trephine. Ultrathin gelatine encapsulation was used on some constructs for protection of the cells. Two surgical procedures were performed, firstly, a 3-port 23-gauge vitrectomy was performed on ex vivo porcine eyes and the TEC was inserted subretinally using a custom instrument and forceps. Secondly, a 16-gauge chute was used transclerally for construct delivery. Morphology and distribution of cells were assessed pre- and post-implantation via phalloidin and DAPI staining. The use of strips rather than circular membranes and pre-cut membranes before cell culture resulted in the least disturbance to cell morphology. The ultrathin gelatine encapsulations have provided a protective effect on the cells. Using the transcleral chute provided better maintenance of cellular morphology and show less cell damage than without. This study has provided us with a better understanding towards development of a surgical technique for subretinal transplantation for translational in vivo studies.
Self-Assembled Bioactive Peptide Nanofibers Promote Corneal Stroma Regeneration

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Corneal diseases are among the most frequent causes of blindness worldwide. In cases of loss of transparency and advanced deformity of the cornea, the current therapeutic option is corneal transplantation which has its limitations. Peptide nanofibers forming hydrogel systems offer a promising approach for regenerative medicine studies because of their biocompatible and biodegradable properties. Moreover, they can be engineered to include bioactive sequences which mimic the active epitopes in the natural extracellular matrix. Here, we utilized bioactive peptide amphiphile (PA) molecules for corneal regeneration in vitro and in vivo. Our results showed that PA nanofibers promote keratocyte response and cornea repair in the fibrotic area of corneal stroma. These nanofibers can be used as an efficient scaffold for keratocytes and thus offer a new platform for corneal tissue engineering.
Atraumatic RPE Debridement with a Novel Instrument Aided by Subretinal Viscoelastics

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Replacing diseased retinal pigment epithelium (RPE) can potentially cure age-related macular degeneration (AMD). Engineered RPE grafts enable cell carrier use to facilitate surgical handling and transplant function. Candidate patients with dry AMD typically have an intact RPE, and transplants "on top" of the host RPE may trigger unwanted cell signaling. We hypothesized that removal of host RPE in the transplantation bed may facilitate graft metabolism and anchoring through tissue engineering at the Bruch’s membrane/choriocapillaris interface (BM/CC). We designed a device where a loop folded within a 20G oval nozzle, widens and moves forward upon release to debride the targeted RPE surface. The instrument was tested with 3 prototypes in 16 eyes of a total 16 rabbits. Following vitrectomy and bleb retinal detachment, the loop was inserted into the subretinal space. The immediate effects of this surgical maneuver were assessed by perfusion fixed histology. A loop made of prolene suture material resulted in consistent RPE debridement by 3 surgeons. Subretinal injection of ≥ 0.25 % hyaluronan (Gelbag®, C. Zeiss Meditec) stabilized the neural retina during loop maneuvering. Visible intraoperative hemorrhages were not observed. A detailed histopathologic analysis revealed occasional small blood clots within the CC, minuscule breaks in BM, yet otherwise intact blood vessels. Taken together, our prototype instrument seems to enable selective and atraumatic RPE removal in rabbits.
In vivo Evaluation of a Fibrin-Agarose Bioengineered Human Lamellar Cornea Substitute. A Preclinical Study

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In vivo analysis of bioengineered human cornea models is a crucial preclinical requirement to assess the usefulness of these models prior to clinical use. In this work, we evaluated a fibrin-agarose biological cornea model in laboratory rabbits to determine the regeneration potential of these artificial tissues in the eye surface. Bioengineered human anterior lamellar corneas were developed in the laboratory using fibrin-agarose biomaterials with human keratocytes immersed within as stroma substitute. Then, human cornea epithelial cells were subcultured on top of the scaffold to generate a bi-laminar cornea. These lamellar corneas were implanted in vivo in New Zealand rabbits by anterior lamellar keratoplasty and analyzed using optical coherence tomography (OCT), optical and histological methods. The bioengineered lamellar corneas showed adequate optical behavior and became completely transparent after 12 weeks in vivo. Furthermore, histological sections showed a well-developed corneal stroma and proper integration in the host cornea after 6 months as determined by histology and OCT. These results suggest that the human lamellar corneas described here were highly biocompatible. The integration observed in the in vivo analysis demonstrated the suitability of the grafted constructs. Fibrin-agarose corneal scaffolds could have potential usefulness for the generation of artificial human corneas for clinical and experimental uses.

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In Vitro Simulation of Corneal Epithelium Microenvironment Induces a Corneal Epithelial-Like Cell Phenotype from Human Adipose Tissue Mesenchymal Stem Cells

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While bone marrow-derived mesenchymal stem cells (MSCs) can acquire certain characteristics of corneal epithelial cells, subcutaneous adipose tissue (AT) is more readily available and accessible and can be harvested by minimally invasive methods. The aim of this study was to determine if extraocular human AT-derived MSCs (hAT-MSCs) can acquire in vitro some features of corneal epithelial-like cells. hAT-MSCs were isolated from human lipoaspirates and expanded up to 3-4 passages. We studied the immunophenotype of MSCs and demonstrated its multipotent differentiation capacity. To test the capacity of differentiation of hAT-MSCs towards corneal epithelial-like cells, hAT-MSCs were cultured on substrata of plastic or collagen IV. We used basal culture medium (BM), BM conditioned with human corneal epithelial cells (HCEcBM), and BM conditioned with limbal fibroblasts (LFcBM). The hAT-MSCs incubated for 15 days with HCEcBM acquired more polygonal and complex morphology as evaluated by phase-contrast microscopy and flow cytometry. Additionally, the expression of transforming growth factor-β receptor CD105 and corneal epithelial marker CK12 got increased as evaluated by flow cytometry, real-time reverse-transcription polymerase chain reaction, western-blot, and immunostaining. These changes were absent in hAT-MSCs incubated with BM or LFcBM. This is the first time that extraocular hAT-MSCs have been successfully induced to acquire certain features of corneal epithelial-like cells by subjecting them to an in vitro microenvironment containing signals provided by differentiated corneal epithelial cells. Our results suggest that hAT-MSCs could provide a novel source of stem cells for patients with ocular surface failure.
Generation of Human Corneal Endothelial Cell Sheets Using Tunable Cell Culture Substrates

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The corneal endothelium forms the inner cell monolayer of the cornea and sustains corneal transparency. Diseases or injuries of the corneal endothelium can lead to severe vision impairment and may require donor corneal transplantation. Peri- and post-surgical endothelial cell loss impedes transplant survival. Therefore, one prioritized aim in corneal tissue engineering is to generate transplantable human corneal endothelial cell (HCEC) sheets with high cell density. Thermo-responsive cell culture carriers are widely used for non-enzymatic harvest of cell sheets. Here we present a novel thermo-responsive carrier based on simultaneous electron beam immobilization and cross-linking of poly(vinyl methyl ether) (PVME, temperature-responsive component) blended with the alternating copolymer poly(vinyl methyl ether-alt-maleic acid] (PVMEMA, peptide binding component) on polymeric surfaces. This approach allows to adjust layer thickness, stiffness, switching amplitude, and functionalization with bioactive molecules to meet the specific demands of HCEC, which require elaborate cell culture conditions and are strongly adherent to the substratum. PVME-blend-PVMEMA supported in vitro cultivation of HCEC and formation of morphological prerequisites for tissue function. Layer stiffness and adhesion-promoting ligand density serve as effective parameters to adjust adhesion and detachment of HCEC sheets. The developed method to engineer corneal endothelial tissue may significantly improve therapeutic options.
A Multilayer Collagen Scaffold for Corneal Stroma Tissue Engineering

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The lamellae forming the natural corneal stroma are parallel to the corneal surface and orthogonal to adjacent lamellae. The 3D construct consisted of a stack of collagen films micropatterned to guide the keratocytes to simulate the composition and the microstructure of the natural stroma. Collagen films were prepared by solvent casting over a micropatterned silicon wafer. The films and 3D constructs were crosslinked by dehydrothermal (DHT) treatment (105 oC, 140 oC, or 150 oC) for 24 h. Films crosslinked at 150 oC for 24 h (DHT150) yielded the best results after enzymatic degradation (in collagenase type II, for 2 h) and in situ degradation studies (in PBS pH 7.4, at 37 oC, for 4 weeks) and the resolution of the patterns was not disturbed after these treatments unlike treatments at other DHT temperatures. DHT150 was therefore, used in the rest of the studies. Human keratocytes (p5-13) were seeded on the patterned films and it was observed that the cells were aligned on Day 1. The cells and their nuclei were elongated along the grooves and the aspect ratio of the nuclei was significantly increased (from 1.66 ± 0.28 to 3.01 ± 0.60 in 21 days). The number of cells was increased 17 fold on single layer films, however, this increase was lower on the multilayer films (7 fold) in 21 days of culture. The lower cell number on 3D constructs was probably due to poor oxygen levels between the layers which led to a decrease in metabolic activity. Transparency of the films was increased significantly during a 30 day culture (77% and 92%, Day 0 and Day 30) at 700 nm and this was comparable to that of native cornea (Fig. 1). The scaffolds appear to have a significant potential to serve as a stroma equivalent and dynamic culturing is necessary to achieve better oxygen and nutrient transport.

Figure 1: Transparency of the collagen films and the native cornea
A Bilayered Electrospun Scaffold for the Corneal Stroma: Effects of Fibre Diameter and Three-Dimensional Environment on Cell Phenotype

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Donor shortages and graft rejection have prompted the search for a tissue-engineered alternative to corneal allografting. In this study, a bilayer of electrospun poly (lactide-co-glycolide) micron and sub-micron diameter fibres was proposed to mimic the macro-anatomical features of the anterior cornea. The dual layer was composed a porous microfibrous layer to allow infiltration of keratocytes and a dense layer of sub-micron diameter fibres to act as a pseudo basement membrane to support an epithelium. Primary human corneal stromal stem cells (hCSSC) and SV40-immortalised human corneal epithelial cells (ihCEC) were cultured on the scaffolds and proliferation (Alamar blue), viability (LIVE/DEAD), and phenotype (SEM, ICC, flow cytometry) assessed. Scaffolds supported cell adhesion and proliferation. Formation of a confluent monolayer of ihCEC was shown on the close-packed sub-micron fibres. The microfibrous layer allowed ingrowth of hCSSC and promoted the keratocyte phenotype evidenced by cell morphology, cytoskeletal organisation, and expression of keratocyte markers including ALDH, Keratocan, and CD34. Cells grown on glass controls had a fibroblast or myofibroblast repair phenotype. Thus, in our hands, the provision of a 3D microenvironment with important topographical cues had the ability to retain the keratocyte phenotype in vitro. In conclusion, a bilayered scaffolding approach may be appropriate for the cornea; a microfibrous scaffold or ‘stromal-type’ environment to allow infiltration of keratocytes and a dense fibrous layer as a pseudo basement membrane on top to promote rapid re-establishment of the corneal epithelium. Finally, co-culture resulted in a structure reminiscent of the native anterior cornea.
Biofabrication of Tissue Spheroids Encaged in Interlockable Microscaffolds

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The combination of bottom-up directed tissue self-assembly approach with classic solid scaffold-based approach is a novel strategy in tissue engineering. Interlockable microscaffolds or "lockyballs" enable rapid assembly of rigid tissue spheroids into 3D tissues in situ. The cellularization of lockyballs or biofabrication of tissue spheroids encaged into lockyballs is an important step in the development of this technology. Lockyballs have been designed and fabricated using two photon polymerization of photo-sensitive hydrogel. The non-adhesive agarose hydrogel and silicone molds have been used to fabricate hydrogel with recessions. Lockyballs have been placed into recessions and suspension of chondrocytes has been added to fabricate encaged tissue spheroids. Microscopical analysis has been used to characterize tissue spheroids encaged into lockyballs. The interlockable microscaffolds or lockyballs have been designed and fabricated using two photon polymerization of photo-sensitive hydrogels. It has been shown that interlockable microscaffolds or lockyballs could be cellularized in recessions of non-adhesive hydrogel. The resulted viable tissue spheroids or chondrospheres encaged into lockyballs with tunable material properties can be interlocked and, thus, enable rapid in situ biofabrication of 3D cartilage tissue. Interlockable microscaffolds or lockyballs with tunable material properties can be cellularized and viable tissue spheroids encaged into lockyballs could be biofabricated. Cellularized interlockable microscaffolds or tissue spheroids encaged in lockyballs is a novel platform technology in tissue engineering.
The only definitive treatment for end-stage liver disease is orthotopic liver transplantation and it is limited by the shortage of available donor organs. To address the issue, we developed a novel method to prepare transplantable liver grafts using perfusion decellularized liver matrix (DLM). While the engineered liver grafts were functional in vitro and could be transplanted in rats, the survival time of the recipient animals was significantly low due to complications related to the high thrombogenicity of the grafts. An ideal engineered liver graft should enable undisrupted blood circulation through the graft in order to allow for its therapeutic testing in a liver failure model. Therefore, we hypothesized that the deposition of an anti-thrombogenic molecule on the DLM scaffold surface will prevent blood clotting when exposed to blood and will prolong the post transplantation survival time of the recipient. Here, we immobilized heparin on the DLM scaffold surface using layer-by-layer film deposition technique. The ability of the heparinized DLM (hDLM) to prevent blood coagulation was tested via ex vivo blood perfusion and there was significant reduction in the blood clot formation in hDLM scaffolds. The macroscopic observation indicated that the extent of reduction was proportional to the amount of heparin deposited. Heparin deposition on the DLM didn’t affect the function of engineered grafts during 5 d of in vitro perfusion culture. Animals receiving the heparinized liver grafts as auxiliary grafts survived for longer than 3 weeks. The generation of transplantable and functional engineered liver grafts that can serve as a reliable alternative for donor organs will offer a new avenue for treatment of liver diseases.
Development of Biphasic Scaffold for Osteochondral Regeneration

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Damage and degeneration of osteochondral tissue at skeletal joints is very difficult to repair due to the avascular nature of cartilage and its limited remodeling capacity, particularly for full-thickness osteochondral defects involving both cartilage and subchondral bone. Existing clinical treatments are aimed at alleviating pain and morbidity in the short term, while long term treatment is rarely successful and often leads to the progression of osteoarthritis. Biomaterials-based osteochondral tissue engineering is emerging as a novel treatment strategy that can address the growing unmet clinical need to develop more effective therapies. The present study describes the development of a novel biphasic scaffold for the regeneration of both cartilage and subchondral bone in full-thickness osteochondral defects, which consists of two different phases with distinct properties tailored to their target tissues. The cartilage phase is a silk fibroin hydrogel, anchored to the bone phase which is a silk-coated biphasic calcium phosphate (BCP-silk) composite scaffold (Fig. 1). The two phases of the biphasic scaffold were shown to imitate the structural characteristics of native cartilage and bone. \textit{In vitro} testing using human bone marrow-derived mesenchymal stem cells over 6 weeks showed that the biphasic scaffold induced tissue-specific differentiation responses in the two phases, indicating its ability to encourage the respective regeneration of cartilage and bone. There is potential for future clinical application of the developed biphasic scaffold in the reconstruction of osteochondral defects that will block disease progression to osteoarthritis.

Fig. 1. Developed biphasic scaffold for osteochondral regeneration.
Surface Modified 3D Porous and Nano-structured Polyetheretherketone (PEEK) for Bone Tissue Engineering

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Porous biomaterials with three-dimensional (3D) surface structure can enhance biological functionalities especially in bone tissue engineering. However, it has been difficult to accomplish this on polyetheretherketone (PEEK) due to its inherent chemical inertness. In this study, a 3D porous and nanostructured network with bio-functional groups was produced on PEEK surface by sulfonation and subsequent water immersion. Two kinds of sulfonation-treated PEEK (SPEEK) samples, SPEEK-W (water immersion and rinsing after sulfonation) and SPEEK-WA (SPEEK-W with further acetone rinsing) were prepared. The surface characteristics, in vitro cellular behavior, in vivo osseointegration, and apatite-forming ability are systematically investigated by X-ray photoelectron spectroscopy, Fourier transform infrared spectroscopy, scanning electron microscopy, cell adhesion and cell proliferation assay, real-time PCR analysis, micro-CT evaluation, and immersion tests. SPEEK-WA induced superior pre-osteoblast functions including initial cell adhesion, proliferation, and osteogenic differentiation in vitro as well as apatite-forming ability and substantially enhanced osseointegration in vivo (Fig.1). Although SPEEK-W presented a similar surface morphology and chemical composition as SPEEK-WA, its cytocompatibility was inferior due to residual sulfuric acid. Our results indicated the cell functions, bone growth, and apatite formation on the SPEEK surfaces were controlled by multiple factors, which included 3D porous and nano-structure, SO₃H groups and local pH environment. In conclusion, surface functionalization and 3D porous networking may broaden the use of PEEK in bone tissue engineering.
Studying Adult Neurogenesis Using a Simplistic Niche Model

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In recent years the natural phenomenon of adult neurogenesis (i.e. generation of new neurons in adult brain) gained increasing importance as a therapeutic approach to treat neurodegenerative diseases. However, current research efforts are confronted with a complex multivariate system regulating the activity of neural stem cells inside the hippocampal stem cell niche. Thus, ongoing studies are focused to unravel the complex interplay of the different exogenous signals involved in adult neurogenesis. We utilize a powerful hydrogel platform based on star-shaped poly(ethylene glycol) and heparin that is capable of controlling a wide range of niche parameters independently to allow for screening of the individual exogenous cues. As such, the material allows to vary the stiffness, degradability and ligand density independently. This powerful matrix variability is combined with advanced life cell microscopy and fluorescence expressing neural cell lines. Specifically, we use βActin-GFP for morphology monitoring and a novel double-transgenic Fluorescent Ubiquitin-based Cell Cycle Indicator (FUCCI) for online cell cycle tracking. By correlating the observed cell behavior with the provided material-based exogenous cues, we hope to gain further insights into the complex regulatory network existent in the neurogenic stem cell niche.
Disc herniation is believed to be the primary instigator of back pain due to the protrusion of nucleus pulposus material into the outer layers of the annulus fibrosus (AF) which contain sensitive nerves. At present there is a lack of treatments to repair damaged AF tissue. Developing biomaterials that can be easily delivered to the damaged AF and promote neo-tissue formation is a key challenge. In this work we developed a porous alginate scaffold with shape memory property which can be delivered using minimally invasive approaches and recover its original geometry once hydrated. Sodium alginate (2% w/v) was covalently cross-linked with various adipic acid dihydrazide (AAD) densities (5, 15, 25, 35 and 45%) by carbodiimide chemistry. A freeze drying step (-30°C) was subsequently performed to impart scaffold’s porosity. Compression tests were performed and compared to native porcine AF tissue. Results showed that the porous alginate scaffold exhibits memory-shape recovery, swelling ratio and mechanical behavior that can be modulated depending on the alginate and cross-linker (AAD) concentrations (Fig. 1A). The scaffold can be repeatedly compressed and expanded without altering its original size and mechanical properties. It provides the potential to deliver the biomaterial directly into the damaged area of the AF tissue, independently of the size and irregular shape of the defect, through a minimal invasive technique (Fig. 1B). Current studies are investigating the potential of this scaffold in an ex-vivo organ culture model.

Figure 1: (A) Equilibrium modulus of scaffolds as a function of AAD concentration compared to native AF tissue in compression test. (B) Swelling behavior of the scaffold after injection in a model of degenerated AF.
Computational Methodology to Determine Fluid Related Parameters of Non Regular Three-Dimensional Scaffolds

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These parameters are not available in all labs. However, fluid parameters should be known prior to other types of experiments. The present work compares an experimental study with a computational fluid dynamics (CFD) methodology to determine the related fluid parameters (k and WSS) of complex non regular poly(L-lactic acid) scaffolds based only on the treatment of microphotographies images obtained with a microCT (μCT). Medical grade poly(L-lactic acid) with a viscosity of 1.8 dl/g and an average molecular weight of 165446 Da was used to fabricate the scaffolds by the freeze extraction and particle leaching process. Different solutions of PLLA in 1-4 dioxane (at 10, 15 and 18 wt.% of PLLA) were homogeneously mixed with PEMA spheres (with diameter ranging from 120 to 200 μm) in mass proportion 1:1. To determine a relation between interconnected porosity and pore size, a permeability test has been developed under the Darcy Law and is available.\textsuperscript{1-3} For each polymer concentration five samples were tested. Cylinders of 6 mm diameter and 3.11 ± 0.17 mm thickness were used. In accordance with the experimental protocol, the fluid flow through the scaffold was varied by controlling the flow rate (20, 40, 60 ml min\textsuperscript{-1}). The total pressure drop across the scaffold sample (ΔP) was measured in each case. The obtained ΔP was averaged out to determine the permeability of the structure using Darcy’s Law. Microtomography was carried out to define the trabecular network and pore distribution, as well as their uniformity in the 3D structure.\textsuperscript{4} The DICOM image files provided by the μCT (348 files for each sample), were the main input for building the geometric model of the scaffold (STL files). The selected volume for reconstruction must be representative of the entire sample, 7.06 mm\textsuperscript{3} (3 mm diameter and 1 mm thickness). The volume segmentation was made with Mimic.

Three different scaffolds geometrical models (STL files), were inserted in a CAD-built test chamber and meshed with Ansys Icem CFD.\textsuperscript{6} About 5 millions cells were defined for each scaffold, using tetrahedral elements. For each scaffold 10 flow rates were tested, representing a total of 30 CFD analyses. To predict the pressure and velocity fields inside the scaffolds, the Ansys CFX code was used to set up and solve the fluid dynamic problem. The culture medium was regarded as an incompressible and homogeneous Newtonian fluid.

A change in the polymer concentration (wt.% of PLLA) determines the trabecular network structure and establishes its correlation with the uniformity and pore distribution. In our working range, an increase in the wt.% PLLA increases the uniformity of the scaffolds, because for large concentration of solvent irregular swelling of porogen spheres occur and zones with large pores, defects and broken trabeculae are found. Increasing the wt.% PLLA leads to a reduction in the structure porosity and the mean pore size.

From the experimental study, it can be deduced that the total difference pressure (ΔP) increases when the wt.% PLLA rises (see figure 1), whereas the intrinsic permeability (k) shows the opposite tendency. The results of the computational simulations confirm these trends (see figure 1). In particular it can be seen, as expected, that increasing the flow results in an increase in ΔP.

The previous results showed only small discrepancies between the experimental and CFD data for high flow rates through the 3D structure. Additionally, a second experimental and
CFD study was performed to evaluate the behavior of the 3D structure at low flow rates (1.5, 3.0 and 4.5 ml min\(^{-1}\)). For low flow rates the accuracy of this prediction reduces. The study of the interconnected structure under different flow rates was used to determine the wall shear stress (WSS). An increase in the flow rate increased the WSS on the trabecular network structure (see figure 1). The WSS spatial distribution along the scaffold was strongly dependent on the complexity of the porous structure, as expected. Overall, the level of WSS found throughout the surface was low and heterogeneously distributed. As is usual, low WSS values were due to the effect of the flow separation regions along the porous sections. For this reason, these regions are characterized by relatively low velocities. In contrast, higher WSS values are due to the impact of the flow to the scaffold. Inside the scaffold, the high WSS values are due to the local acceleration of the flow along a single pore just before the flow separation. An optimal reconstruction model for 3D complex geometry, appropriate pre-processing and image analyses, and the computational fluid dynamics methodology implemented in this study could be used as alternative tools to assess various mechanical variables for scaffold structures (porosity, pore size and trabecular network distribution, structural difference pressure, intrinsic permeability and wall shear stress).
Transplantation of insulin producing islets into hepatic portal vein of patients with type 1 diabetes mellitus offers an appealing therapeutic alternative to exogenous insulin administration; but this method suffers from rapid dysfunction and destruction of a major amount of transplanted islets. Thus, extra-hepatic islet transplantation approaches are increasingly being considered as a promising alternative. However, this methodology requires creation of minimally invasive artificial microenvironment that closely mimics natural anatomical context of pancreatic vasculature to maximize graft survival and host reintegration. To this end, in the current study, we developed polylactide-based biocompatible highly porous scaffolds with controlled morphology using a modified-thermally induced phase separation process. The macro shape of the scaffold resembles an open-capsule with a void volume of about 0.25 cc, and with a wall thickness of about 600 µm. The capsule wall possesses radially aligned, interconnected, gradient pores with inner and outer diameters varied from 10 to 50 µm respectively. Process parameters, such as quenching and coarsening temperature/ time, polymer concentration and solvent type, were studied in great detail. Current process allows the fabrication of open-capsular scaffolds with controlled shape and with varying pore gradients, inner lumen diameters and wall thicknesses. The channeled structure of the scaffold would augment in vivo vascularization process prior to islet transplantation and facilitate sufficient oxygen supply to transplanted islets. The capsules are implanted both subcutaneously and in to major omentum of rats and vascularization of the graft is being followed using magnetic resonance imaging in preliminary in vivo tests. (Supported by the Ministry of Education, Youth and Sports of the Czech Republic, grant No. CZ.1.07/2.3.00/30.0029)
A new and powerful approach for designing hydrogels for cartilage regeneration is the self-assembly of supramolecular building blocks to form macroscopic materials. Self-assembly is recognized as a process in which small molecules organize into well-ordered structures by reversible, non-covalent interactions. We report the study of two different hydrogels based on the supramolecular aggregation of a) ureido-pyrimidinone (UPy) and b) bisurea (BU) modified poly(ethylene glycols) (PEGs) as self-assembling 3D scaffolds for cartilage regeneration. Stability and biocompatibility of the materials were assessed during 28 days, including analysis of cell viability, morphology, and distribution, and matrix deposition. Chondrocytes suspended in culture medium were added to freeze-dried and pulverized 10 wt-% BU-PEG and UPy-PEG. These mixtures self-assembled to form stable hydrogels, which were then cultured for 28 days in chondrogenic differentiation medium. Depending on the composition, self-assembled BU-PEG and UPy-PEG hydrogels remained stable for 28 days in culture. Both materials supported a rather uniform cell distribution, cell viability of >95% and retention of the rounded chondrocyte morphology during the entire culture. Currently, matrix deposition by the cells is being evaluated. In conclusion, it is shown that self-assembled supramolecular BU-PEG and UPy-PEG hydrogels were not only able to withstand cell culture conditions for up to 28 days in vitro but also provided an environment for chondrocytes to survive and retain their natural phenotype. Overall, these results show that these supramolecular self-assembling hydrogels provide a promising platform for cartilage regeneration.
Fibrous Alpha Alumina Scaffolds for Bone Tissue Engineering Applications

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Green alumina based fibers were fabricated by wet spinning through ionotropic gelation. Prior to fibers fabrication, alumina slurry was prepared by mixing aluminum hydroxide (Al(OH)₃) with 4 wt% chitosan solution in 2% acetic acid medium in 1:3 weight ratio. The viscosity of the alumina slurry was optimized by rheological study and the gel formation with STPP was analyzed by the gelation kinetics study. The well mixed alumina slurry was spun into fiber by wet spinning in sodium tripolyphosphate (STPP) bath through ionotropic gelation of chitosan. The structure and morphological features of the fibers were characterized by Scanning Electron Microscopy (SEM) and X-Ray radiation analysis. The average diameter of the produced green fiber was measured to be ~ 120-150 µm prior to sintering and was flexible under wet condition. Alpha alumina fibers were formed when the fibers were calcined at 950 ºC for 2h. After heat treatment of 1550 ºC for 2h, the fiber had hexagonal platelet morphology with grain size ~ 4.5 µm. Crystallite size of alpha alumina varied between 46 nm to 65 nm. The sintered fibrous alumina scaffolds produced were found to have interconnected open porous structure with a compressive strength similar to cancellous bone and favorable for tissue ingrowth while implanted in vivo. The response of MG-63 cells, to these scaffolds was evaluated using assays of MTT and alkaline phosphatase (ALP) activity. Results indicated that alumina fibrous scaffolds provided a favorable substrate for the growth and differentiation of osteoblasts cells and a promising scaffold for bone tissue engineering applications.

Key words: Alpha alumina, Ceramics fiber, Ionotropic gelation
Patient Compliant Biodegradable Urinary Stents

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A urinary stent is a thin tube, which is inserted in the ureter to prevent or treat the obstruction of urine flow from the kidney. Silicone, latex, PVC and polyurethanes are the most widely used materials for the preparation of stents. Nonetheless, severe clinical complications may result from the use of these materials such as, fracture, encrustation and infection. In some cases, the stent is temporary and it is often required a second surgery to remove it. In this work, we developed hollow tubes (stents) from natural origin polysaccharides. Polymeric solutions were injected into templates of appropriate geometry and cross-linked at room temperature with CaCl2 or KCl. Hollow tubes, with a diameter of 2 mm, were prepared and characterized in terms of surface morphology by SEM. Water uptake and polymer degradation studies were executed using an artificial urine immersion solution. The developed stents presented high water uptake ability but are able to maintain their shape and integrity. The degradation timeframe can be tuned to occur between 14 and 60 days. In vitro assessment of possible encrustation, i.e. the deposition of Mg and/or Ca salts, was also carried out using SEM-EDS and no encrustation was observed for the tested time periods (up to 28 days). The ability to avoid bacterial adhesion and the creation of a biofilm was evaluated with gram-positive Staphylococcus aureus and gram-negative Pseudomonas aeruginosa and we obtained results comparable to commercially available stents. Cytotoxicity and cell adhesion studies were also executed to compare the developed materials with a commercial stent. The results obtained reveal that the proposed materials are major breakthroughs in the development of biodegradable stents.

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Biomaterial Degradation and Complement Activation in a 3D Soft Tissue-like Model

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Biomaterial degradation and their effects on tissue inflammation are usually tested in vivo, and so effective 3D in vitro screening would be of great value for biomaterial development. We describe two 3D models based on plastic compressed (PC) collagen hydrogels which provide a soft-tissue like environment for pre-in vivo testing of biomaterials. In the first model, polyglycolic acid (PGA) was allowed to pre-degrade (hydrolysis) in media for up to 28 days; and this PGA-media was incorporated into a PC gel between two other PC layers containing human dermal fibroblasts. Live dead cell staining determined the cell death as a result of degradation products during subsequent incubation over 24 hours. Cell death was detected. A second model of inflammation was based on responding collagen gel layer seeded with whole blood. Feasibility tests showed that compressed collagen alone produces a modest baseline inflammatory response as activation of complement system in whole blood, and platelet binding was low. In this configuration one layer was seeded with whole (sheep) blood adjacent to PC layers containing a) fibroblast as potential sources of inflammatory cytokines and b) partly degraded PGA. This model configuration was made rapidly and the three layers segregated as predicted and yet remained physically attached throughout the culture. Together these 3D tissue mimic models provide the potential for in vitro screening of large number of biomaterials prior to animal testing.
Stents are commonly used to widen hollow organs affected by stenosis. We developed the BioStent based on a self-expandable stent structure which is integrated in a vital and autologous tissue engineered construct. This concept excludes damaged tissue from the luminal side and provides a functional cell layer instead. Initially, we adopted the BioStent concept for vascular application: a self-expandable, warp-knitted nitinol stent served as supporting structure that was covered by an autologous tissue engineered vessel graft. The vascular BioStent was successfully implanted in a sheep model. This platform technology now serves as the basis for the development of an airway stent - the PulmoStent - to overcome current limitations of airway stenting to treat stenoses caused by bronchial carcinoma. Respiratory epithelial cells on the luminal side might allow better transport of mucus. Additionally, a separating layer from polyurethane and embedded microparticles which contain tumor-specific drugs inhibit restenosis by bronchial carcinoma. Culturing respiratory epithelial cells on the inside of the stent required the successful development of a new bioreactor system which allows an air-liquid-interface culture. In vitro culture of respiratory epithelial cells from sheep was also established as a first step towards an implantation in a large animal model.
Decompression sickness (DCS) is a potentially fatal condition caused by the formation of inert gas bubbles in tissues which has been subjected to a rapid reduction in ambient pressure. DCS results from the response of organ systems to these bubbles. Initially, relatively mild symptoms occur in the skin, lymphatic or musculoskeletal systems; these may be followed, by more severe effects in the central nervous system. Susceptibility to bubble formation and growth in different tissues varies, but little is known about how this relates to the clinical progression of DCS[1]. Difficulties in collecting relevant experimental data are a major obstacle to furthering our understanding of DCS. By engineering Collagen type I hydrogels, with varying density and biomimetic matrix composition, we have developed an \textit{in vitro} system for studying the condition[2]. We have varied specific parameters we hypothesize to be of significance to bubble dynamics, namely, tissue elasticity and anisotropy, cell type and perfusion level. A pressure vessel we have designed, allows for the microscopic observation of bubble dynamics in these constructs during a simulated dive. This system enables a systematic and quantitative investigation of the tissue parameters that affect bubble growth. In parallel with this a mathematical model of bubble dynamics in soft tissue has been developed. Using data derived from cavitation rheology[3] experiments, the model can be used to simulate the same dive profiles as the pressure chamber, providing a direct comparison to the experimental results. Initial bubble growth shows a dependence on tissue elasticity, as controlled by matrix density; and anisotropy, as controlled by cyclic loading.
3-Dimensional Agent-based Model to Investigate the Effects of Scaffold Degradation on Angiogenesis

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Modeling and simulation frameworks built upon sound biological knowledge and novel computational strategies have become a valuable tool for understanding the underlying mechanisms of biomedical systems, leading to improved theoretical knowledge and enhanced experimental strategies. In this work, vascularization of degradable biomaterial structures used as tissue engineering scaffolds has been investigated using an agent-based model (ABM). Tissue engineering scaffolds are used as a physical support structure and regulator of cellular activities in a wide range of biomedical applications, including production of functional implants for regenerative medicine. Scaffold vascularization is essential for transferring oxygen and nutrients to the tissue cells growing inside scaffold structure, and has been a major limiting step in tissue regeneration, largely restricting clinical application of these biomaterial structures. Microstructure of biomedical scaffolds is characterized by parameters such as porosity, average pore size, interconnectivity, and pore shape as well as mechanical properties such as Young’s modulus which change dynamically with the degradation of scaffold. Predicting the degradation behavior of biomaterials allows optimization of the tissue engineering scaffolds. By integration of a model that enables predicting the degradation of the porous hydrogel scaffold, it would be possible to study the effect of scaffold degradation on angiogenesis and tissue growth. Since the degradation model is based on fundamental parameters that are taken directly from the physical system, it would be possible to use the model for different types of hydrogels that are degraded via similar bulk erosion mechanism.

The developed framework is implemented in Java, using Repast (Recursive Porous Agent Simulation Toolkit) which is an open-source agent-based modeling and simulation platform. Software agents are developed as independent computational entities to represent endothelial cells (ECs). These agents interact together and with their microenvironment, leading to formation of new blood vessel capillaries and invasion into deeper parts of scaffolds. An embedded rule base governs the behavior of individual EC agents. ECs are capable of sensing their microenvironment to perceive the location of other neighboring agents and the geometry of surrounding scaffold, as well as concentration and gradients of the soluble and insoluble factors, and then performing various actions such as elongation, proliferation, and sprouting or branching. In each capillary branch, only the leading EC, referred to as tip cell, is active and capable of elongation, migration, and proliferation. Other ECs in each branch are referred to as stalk cells, and are only activated randomly during simulation to perform sprouting, if other conditions in their environment allows them to do so.
3D scaffold models with homogeneous and heterogeneous spherical pores are developed to investigate the impact of scaffold mean pore size and interconnectivity on rate of vessel growth and the developed blood vessel density. Different pore sizes can be used as model input and for each pore size, various pore interconnectivities are studied. Simulation results for homogeneous pore structures support the positive effect of increasing pore size and interconnectivity on angiogenesis. The model is used to understand the effect of degradation dynamics on scaffold vascularization. Our initial results indicate that the effect of scaffold degradation becomes significant at low porosities. A number of experimental studies are being undertaken to investigate these findings. Controlling blood vessel assembly by modulating EC behavior with changes in the characteristics of the extracellular environment is critical to development of replacement tissues. The model predicts behavior of blood vessel networks growing inside a matrix towards regions with higher concentration of growth factors, as expected from a developing blood vessel network and comparable to experimental findings. The computational and experimental results can be used to guide the design of optimized porous scaffolds that degrades over time.
Engineered 3D in vitro Model Tissue System to Assess Nanoparticle Fate

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Nanoparticulate delivery systems are normally tested on 2-dimensional (2D) cell cultures, but oftentimes positive results do not translate well in vivo, leaving a need to develop more physiological 3D tissue models. Here we present results of a novel 3D model, using plastic compression (PC) of collagen to form depots of hyaluronan nanoparticle (HA-NP) which can be used to determine their tissue fate. The aim here was to understand the location and mechanism of NP entrapment following compression. 0.1 mg/ml HA-NP (FITC tagged; around 40 nm diameter) was added to collagen solution prior to gelation. Absorbent plungers on top of the gels produced compression in 24-well plates (16 mm diameter). Histological sections of PC collagen-NP constructs were analysed by fluorescence microscopy with intensity scans across the sections of 1.0, 1.5 and 2.0 ml (initial volume) PC constructs, to quantify trapped HA-NP distribution. In 1.0 ml constructs, a HA-NP depot was formed at the top, fluid leaving surface (FLS), whilst for 1.5/2.0 ml constructs, HA-NP accumulated at both the top and bottom surfaces, consistent with predicted non-linear fluid flow during the compression period. This effect was enhanced when a 2nd collagen layer was added on top of the PC-NP layer and co-compressed. Addition of cells with the collagen further complicated the fluid outflow, limiting the cell density used, but still enabling fabrication of dermal fibroblast tissue equivalents containing the HA-NP biomaterial. This system provides a controllable form of NP depot within a tissue equivalent, for the study of NP fate in 3D.
Natural Architectures as Bio-inspiration for the Development of 3D Scaffolds for Tissue Engineering: Studies on Marine Sponges

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Marine natural products, in particular sponges, are exceptionally rich in natural products and present huge prospective for biomedical applications. We have focused on the potential of biosilica from Petrosia ficidormis for novel biomedical/industrial applications. A bioceramic structure from this sponge was obtained after calcination at 750°C for 6 hours in a furnace. The morphological characteristics of the 3D architecture were evaluated by scanning electron microscopy and micro-computed tomography revealing a highly porous and interconnected structure. The skeleton of Petrosia ficidormis is a siliceous matrix composed of SiO2. Induction of bioactivity was attained by subjecting the bioceramic structure to an alkaline treatment (KOH 2M and 4M) for 1 and 3 hours. In vitro bioactivity of the bioceramic structure was evaluated in simulated body fluid (SBF), after 1, 3 and 7 days. Observation of the structures by SEM, coupled with spectroscopic elemental analysis (EDS), has shown that the surface morphology was consistent with a calcium-phosphate CaP coating, similar to hydroxyapatite (HA). The determination of the Ca/P ratio, together with the evaluation of the characteristic peaks of HA by FTIR and XRD, has proven the existence of HA. In vitro biological performance of the structures was evaluated using an osteoblast cell line. Cells were seeded on the bioceramic structures and their morphology, viability and growth was evaluated by SEM, MTS assay and DNA quantification, respectively. In vitro test results demonstrate that cells are able to grow and colonize the bioceramic structures.

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The Therapeutic Effect of the Mesenchymal Stem Cells / Small Intestine Submucosa Composite Gel on the Regeneration of Vocal Fold

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Vocal fold (VF) scarring is one of the major causes of severe dysphonia. Tissue regeneration requires appropriate cells and a scaffold. Mesenchymal stem cells (MSCs) have great therapeutic potential in wound healing. Small intestinal submucosa (SIS) is composed of various ECM. We hypothesized that when advantages of SIS are ideally combined together with MSCs, they could support the environment for cell survival, matrix synthesis and structural stability, and also induce regeneration of VF defects. The MSCs from rabbit iliac bone marrow were isolated and cultured. Rabbit vocal fold was injured bilaterally by electrocauterization. After injury, MSCs with SIS composite gel (0.1 ml) were injected into the right vocal folds and saline into the left. Two weeks, one month and two months postoperatively, the site was evaluated functionally, radiologically, and histologically. Postoperatively telescopy showed that injected MSCs-SIS were distributed throughout the vocal fold wound site and augmented to volume without inflammation. MSCs-SIS injected vocal folds displayed improved epithelialization compared with sham-treated folds. Histologic analyses revealed favorable restoration of the extracellular matrix and decreased dense collagen deposition in MSCs-SIS injected group. The MSCs were ideally combined with SIS as injectable ECM material and MSCs-SIS injected into a scarred vocal fold enhances healing of the vocal fold in rabbits.
Short peptides have attracted significant attention in recent years as building blocks for the design of novel materials in particular hydrogels. The library of 20 natural amino acids offers the ability to play with the intrinsic properties of the peptide such as structure, hydrophobicity, charge and functionality. The main challenge in this field is to gain control over the physical properties of these materials. This requires an in depth understanding of the self-assembling processes at all length scales. Beta-sheet forming peptides are very attractive due to the “simplicity” and robustness of the structure formed at the molecular level and the ease of functionalisation. We have recently developed a variety of functional and responsive hydrogels exploiting the self-assembly of a family of short beta-sheet forming peptide (4-10 amino acid) based on the alternation of hydrophobic/ hydrophilic amino acids. Here we will discuss the design principles allowing the creation of hydrogels with tailored properties, this includes the design of injectable, sprayable and responsive hydrogels. We will then discuss the use of these materials as a technological platform for cell/ stem cell culture, encapsulation and delivery.
This work is focused on the use of pectin, a negatively charged polysaccharide consisting in a linear backbone of unbranched homogalacturonan residues alternately linked to branched rhamnogalacturonan I and II residues. Water-insoluble gels may be obtained with the use of divalent or trivalent cations. Recently, our studies are focused on the production and characterization of different pectin-based gels for regenerative medicine applications. Pectin gels were studied as cell carriers. Macrogels with different rheological properties and microspheres were produced with different degree of crosslinking. The injectability of the gels cross-linked with different salts (CaCl2, CaCO3, ZnCl2 and FeCl3) was tested by rheological and texture analyses. Pectin was chemically modified by RGD grafting and partial oxidation to improve cell adhesion and to obtain faster degradation rates of pectin gels, respectively. MC3T3 preosteoblasts and human mesenchymal stem cells were immobilized in the RGD-pectin microspheres for bone tissue regeneration showing good cell viability, metabolic activity and osteogenic differentiation. In vivo experiments confirmed that RGD-pectin microspheres provided a complete adaptation to bone defect and induced bone regeneration avoiding the dispersion of cells after implantation.

As sterilization is a crucial aspect to be faced for natural polymers, several sterilization methods were investigated. Gamma radiation at low doses resulted the most suitable treatment, avoiding the side effects caused by moist heat and EtO sterilizations.

Overall, our studies show the real potentiality of pectin as novel and versatile biomaterial for regenerative medicine applications.
Effect of a Cell-based Bioactive Smart Patch after Myocardial Infarction in Swine

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Introduction: Heart failure is the end-stage of many cardiovascular diseases, such as acute myocardial infarction (MI), and is one of the most appealing challenges for regenerative medicine. The objective was to develop, apply and monitor on-line a bioactive smart patch with GFP-adipose tissue derived progenitor cells (ATDPCs) after MI in swine. Left lateral MI was induced by coronary artery ligation (treated=8; control=5). Thirty minutes after, the patch with or without GFP-ATDPCs was implanted over the infarcted area. In 2 control and 1 treated pigs the patch was connected to a bioimpedance monitoring system to analyse scar evolution. Electrical impedance spectroscopy (EIS) measurements were obtained during 1 month by a custom implantable system with telemetry. Pigs were sacrificed after 1 month to obtain samples for morphometric and immunohistological analysis. Morphometry revealed tendency, although not statistically significant, to reduce infarct area in treated group compared with controls (10.2% vs. 6.5%; P=0.14). Histopathology analysis confirmed the presence of GFP-ATDPCs in infarcted myocardium and new vessel formation in the bioactive smart patch. Inflammation state assessed by CD3/CD25 ratio showed significant differences between groups (0.6±0.4 and 0.2±0.3; P<0.001). Impedance magnitude at low and high frequency were initially separated and converge after a period of 4.5-7 days, which is coherent with the scar formation. However, no significant differences were found between groups. The smart patch integrated new vessels after implantation and minimized the inflammation in myocardial ischemia. This is the first study demonstrating EIS measurements in vivo.
We previously reported the isolation of porcine cardiac extracellular matrix (pcECM) exhibiting matched myocardial bio-mechanical properties in vitro. Here we further characterize this biomaterial as a patch treatment for acute phase myocardial infarction (MI) in rats. Wistar rats (n=24) were divided into 3 groups: patch implantation after MI, non-treated MI (negative control) and sham operation (positive control). MI was induced by lateral artery descending ligation. Patches were sutured onto the anterior wall of the infarct site. All rats were weighed and echocardiographic and ECG parameters were recorded before and after procedures for up to 4 weeks. During sacrifice cardiac hemodynamics were recorded; carcasses and harvested hearts were histo-pathologically evaluated. Patch treated rats displayed significant improvement (t=30 days, p<0.05) in average percentage weight gain compared with the non-treated controls (135±7% and 119±2%, respectively), similarly to the sham group (130±8%, p>0.05). Concomitantly, cardiac functional parameters of treated rats (ejection fraction, EF; fractional shortening, FS; and fractional area change, FAC) were similar to the sham and significantly better (p<0.05) than the negative control (EF=88.7±2.0, 79.8±6.4 and 49.5±5.1; FAC=86.4±5.2, 90.2±7.6 and 48.0±5.2; FS=80.9±8.2, 135.1±30, and 26.4±8.0, respectively). Additional results of cardiac hemodynamics and histological analyses will be also presented.

Taken together these results affirm the potential of thin acellular pcECM for the possible treatment of MI and end-stage heart failure in patch deposition treatment modality.
In-Vivo Evolution of Collagen Organization in the Stented Vessel Wall of Tissue Engineered Heart Valves

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Bioprosthetic and mechanical valves are the currently available prostheses for heart valve replacement, but they lack growth, repair and remodeling capabilities. Therefore, stented tissue-engineered heart valves (TEHV) are promising alternatives that might overcome these limitations. Understanding the collagen orientation changes under the mechanical stresses applied by the stent is important to gain insight into remodeling of the extracellular matrix (ECM) in the stented vessel wall of TEHV. TEHV, based on rapidly degrading scaffolds, and ovine vascular-derived cells were cultured for 4 weeks in bioreactors and decellularized. In-vivo collagen remodeling was evaluated by quantifying collagen orientation of the explants obtained after 8, 16 and 24 weeks. Collagen tortuosity was quantified using Gabor wavelet by calculating Gabor coefficient in different scales and directions. Immunohistology was performed to evaluate the presence of elastin, collagen type I and III. Results show that at the luminal side of the vessel wall, fibers are aligned in the circumferential direction and the tortuosity is increased with implantation time. In the outer region, where the tissue is in contact with the stent, collagen fibers were aligned in the direction of the struts near the struts and randomly orientated in between the struts. The amount of elastin and collagen III was increased and collagen I was decreased by implantation time. Collagen orientation is to a large extent influenced by stenting. Elastin, which was not present in TEHV at time of implantation, increased significantly after 24 weeks. Collagen tortuosity is increased with implantation time. These observations indicate the development of the engineered vessel wall toward native-like tissue.

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The Effect of Controlled 3D Biomechanical Conditioning on the Orientation of Myogenic Cells in a Myocardial Extracellular Environment

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Although the promising whole-organ TE concept have entered the myocardial TE field already 5 years ago, there still is no known approach to transfer the known beneficial effects of mechanical stimulation of myocardial constructs to the field of whole heart TE. We constructed a bioreactor system specifically aimed at the cultivation of decellularized whole-hearts through that were pre-seeded with myogenic cells, under controlled perfusion and 3D left-ventricular stretching. Hearts were cultivated for 96 hours under 3D left ventricular stretching, controlled at 10 % longitudinal elongation. Constructs cellular viability was observed via a perfusion based WST-1 Assay. Varying cellular alignment was quantified via analyzing the orientation angle and the circularity of nuclei after their exemplary representation by confocal microscopy. We observed a significant increase of cellular viability of the pre-seeded scaffolds for stimulated and non-stimulated hearts. Both confocal microscopy of the pre-seeded cells and continuative quantitative determinations indicated a biological response to the applied biomechanical stimulation that clearly increased the extent of cellular alignment inside of the stimulated constructs. By our applied operational approach we could successfully recover the known beneficial effects of stretching to the cellular alignment of myocardial constructs in whole decellularized hearts that were pre-seeded with myogenic cells. This is the first approach that transfers real 3D stretching to anisotropic three-dimensional ECM based TE constructs.
Hydrogel-based Biodegradable Scaffolds for Cardiac Tissue Engineering

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Disease, degeneration, or dysfunction in the cardiac tissues remains as the number one cause of death globally. For example, myocardial infarction (MI) is one of the most common cardiac injuries. There have been a number of efforts such as cell transplantation strategies to repair the injured myocardium. However, a major drawback of this approach is the high levels of cellular death shortly after the delivery of cell suspensions. This is due to the direct exposure of single cells to the physiological stress conditions and the lack of a three-dimensional biomimetic surrounding. To address this limitation, one can encapsulate cells within 3D flexible hydrogels for transplantation purposes. In this project, we synthesized photolabile methacrylated gelatin (GelMA) to generate biodegradable scaffolds and encapsulated cardiac side population cells (CSPs) within them. We optimized the polymer and photoinitiator concentrations to obtain high levels of cellular viability, spreading, and metabolic activity for CSPs. In addition, the cellular behavior of CSPs was also characterized under physiological stress conditions, such as hypoxia and oxidative stress. Moreover, the potential of this system as an in vivo delivery approach was demonstrated by fabrication of CSP-laden microgel particles. As a result, we demonstrated that GelMA hydrogels with cell interactive functionalities are suitable biomimetic biomaterials as cellular delivery systems for CSPs with possible in vivo applications. Delivery of CSPs within biodegradable hydrogels to the MI tissue may potentially improve the survival of CSPs and promote the regeneration of cardiac tissues.
Elastin Functionalization of a Biodegradable Polypropylene Fumarate Graft for Vascular Applications

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The major problems associated with vascular prostheses are the non-biodegradability of the implant, small caliber related thrombosis and neo-intimal hyperplasia. In this respect, polypropylene fumarate (PPF) may be considered a promising candidate due to its biodegradability and good mechanical properties. Following a bioinspired approach, an elastin coating has been performed onto porous PPF scaffolds with the aim of enhancing endothelial cell proliferation, reducing platelets adhesion, smoothening muscle cell growth and, finally, the overgrowth of neo-intima and thrombosis. Elastin has been suspended in Tris buffer and deposited onto porous scaffolds of PPF, previously prepared by mask projection Excimer laser photocuring. The presence of elastin onto the scaffold after deposition has been evaluated by FTIR and SEM analysis. The biodegradability of the graft was also evaluated keeping it in a buffer solution at 37°C up to 60 days. Endothelial cells have been seeded onto the functionalized scaffolds and their adhesion, proliferation and matrix deposition were evaluated in vitro up to one month of cell culture. FTIR and SEM analyses have confirmed the presence of elastin fiber aggregates bound to the PPF scaffold. In terms of biodegradability, PPF-based scaffolds have shown a mass loss of 25%, revealing good resorbability properties of this material. Preliminary results show an enhanced cellular adhesion and activity onto elastin functionalized PPF scaffolds. In conclusion, a bioinspired PPF-based material was extensively characterized to study its properties of biodegradability and affinity with elastin. The promising results confirm this innovative material as a promising alternative for vascular applications.
Regenerative medicine aims to treat myocardial infarction with stem cell seeded scaffolds. Scaffold selection depends on the adherence of cells, continued vitality of the construct in culture and mechanical properties. In this study a variety of biomaterials such as polyurethane (PU), collagen, TachoSil® and pericardium were seeded with mesenchymal stem cells (MSC) isolated from umbilical cord tissue. The patches were evaluated using H&E stains and scanning electron microscopy (SEM) to investigate the distribution of the MSC in all axes. Furthermore patches stained with Syto9 and propidium iodide were utilized to investigate the vitality. WST-1 mitochondrial assays indicated the level of cell proliferation. PU scaffolds were also tested regarding elasticity at different thicknesses and in seeded vs. unseeded states. MSC were found to distribute well on all scaffolds with distribution dependent on cell seeding density. Collagen could only be seeded evenly up to monolayer (0.375 x 10^6 cells/cm²) while the rest of the scaffolds allowed seeding up to 1.5 x 10^6 cells/cm². Histology and SEM showed that the cells form a confluent multilayer on PU and pericardium; TachoSil® was found to be seeded deep into its pores. WST-1 assays suggested better proliferation on pericardium and PU compared to collagen and TachoSil® at high densities. Adjustment of PU thickness allowed a variation of the E-Modulus towards the level found in the human myocardium (~500 kPa). Furthermore seeding at a density of 0.750 x 10^6 cells/cm² had no significant effect on elasticity. PU and pericardium are two strong candidates for MSC seeding in the framework of cardiac patch development. Both allow high seeding densities and have no detectable adverse effects on the cells.
Microbubble Scaffold for 3D Cardiomyocytes Culture and its Potential for Cardiac Tissue Engineering

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Three-dimensional micro-environment closely mimics the natural extra-cellular conditions. As previously demonstrated, porous scaffolds were fabricated to culture primary murine cardiomyocytes. Both type I collagen and gelatin were used in the production. Collagen is the most abundant protein in mammals, especially in the heart, and is highly relevant to cardiac performance. Gelatin is derived from collagen inside animal skin and bones. Both collagen and gelatin are biodegradable and biocompatible. Regarding cells, primary cells were harvested from neonatal mice. We believe that understanding the behavior of cardiomyocytes in three-dimensional environment will increase the probability of succeeding in myocardial tissue engineering, and even repairing injured hearts. 3D microbubbles composed of gelatin or collagen-doped gelatin were produced by a microfluidic apparatus. Neonatal cardiac cells containing both cardiomyocytes and cardiac fibroblasts, were seeded to obtain healthy in vitro myocardial environments. Cell morphology, contraction frequency, protein expression, and cytoskeleton distribution of both systems (our three-dimensional system versus culture plates) were examined. 3D biomaterial scaffolds generated using a microfluidics technique were tested for murine cardiomyocyte culturing. Cell morphology, contraction frequency, protein expression, and cytoskeleton distribution were examined and compared to the results of cardiomyocytes cultured in conventional 2D culture plates. This investigation demonstrated the potential of 3D biomaterials based cell scaffolds. Cardiomyocyte characteristics were shown to be retained for a prolonged period of time.
Heart Tissue Reconstruction Applying Microstructured Polymer Scaffolds and Autologous Cells in a Rabbit Model

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Heart reconstruction surgery is a complex procedure that requires miscellaneous knowledge and modern techniques. Stem cell therapy and development of artificial tissues are extensively explored practices in this field. The aim of this study was to fabricate a biocompatible scaffold suitable for heart reconstruction. In our experiments, laser microstructuring together with soft lithography were used as the polymer scaffold engineering techniques. Several types of polymers were tested for cell growth and artificial tissue sheet fabrication. According to our data, polydimethylsiloxane (PDMS) was the most biocompatible material, best suited for cell growth. The results demonstrate that a small artificial tissue patch fabricated from microstructured PDMS scaffold seeded with autologous myogenic cells, which was implanted into experimentally injured rabbit heart, had integrated properly. It is known that the main limitation of cytotherapy is poor viability of transplanted cells. In our case, it was found that transplanted cells remain viable throughout the testing period. Moreover, PDMS scaffolds fabricated in such way showed suitable mechanical properties and biocompatibility in experimental rabbit heart lesion. Furthermore, soft lithography significantly improved the fabrication throughput and decreased processing costs as well as enabled the use of polymers which aren’t well microstructurable by laser, such as PDMS. In conclusion, microstructured polymer scaffolds combined with autologous cells could be successfully used for cardiomyoplastic applications.
Stem cell therapy is a promising tool to restore contractile function after myocardial infarction. Unfortunately, clinical trials still show disappointing results with only minor improvements in cardiac function. The major problem of cellular therapy is lack of persistence of sufficient cells at the site of injury, independent of administration route. We designed a novel technique to overcome this problem by directing stem cells to the infarcted area using targeted microbubbles (MB) and ultrasound (US). For this adipose derived stem cells (ASC) were coupled to MBs using an antibody against CD90. This stem cell-bubble complex was named 'StemBell'. StemBells were targeted to the infarcted area via a second antibody on the MB: anti-CD54. US (1 MHz) was applied to exert acoustic radiation force on the StemBells. In vitro we demonstrated, using flow cytometry, that the procedure to create StemBells, as well as exposure to US had no negative effect on cell viability. Binding of MBs to ASC did not affect their ability to attach to a culture dish, demonstrated by light microscopy. In a flow system we showed that US pushed StemBells to the side. In a rat model for myocardial infarction and reperfusion, we demonstrated that 3h after intravenous injection of 1 million DAPI-labeled StemBells ('StB'), significantly more cells (6-fold) were present in the infarcted area (n=6; p<0.01 vs ‘ASC alone’), by performing fluorescence microscopy on heart cryosections. Applying US ('StB+US') even lead to an 8-fold increase (n=6; p<0.01 vs ‘ASC alone’, p<0.05 vs ‘StB’). Notably, retrieved cells coincided with CD54 positive areas.

In conclusion, we successfully demonstrated proof of principle of a novel technique to increase the number of stem cells at the site of injury.
Acute Myocardial Infarction Affects the Number of Stem Cells Obtained from Adipose Tissue, but not their Functional Characteristics

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In almost all pre-clinical animal studies investigating stem cell therapy after an acute myocardial infarction (AMI), the administered stem cells are isolated from healthy donors. In clinical practice, however, patients who suffered from AMI will receive autologous stem cell therapy, for example with adipose derived stem cells (ASC). During an AMI many inflammatory molecules are released into the bloodstream and we hypothesized that this might affect stem cells. This could explain why results obtained from clinical trials differ from animal studies. In this study, we investigated whether there were differences between ASC obtained from rats with an AMI and healthy control rats, both on percentage of ASC and functional characteristics, such as proliferation and differentiation capacity. AMI was induced in rats by a 40 min ligation of the LAD followed by reperfusion. ASC were isolated from inguinal adipose tissue 1 and 7 days post-AMI, and compared with ASC from healthy control rats. We found that significantly less ASC were present 1 day post-AMI in the stromal vascular fraction (SVF), as determined by a colony-forming unit (CFU) assay (p<0.001 vs control, n=6). No differences were observed 7 days post-AMI. These data were confirmed by flow cytometry, showing less cells with stem cell associated markers present in the SVF 1 day post-AMI. Interestingly, proliferation assays showed that there were no differences in population doubling times in the first three passages, as well as no differences were found in cell size and cell volume between all groups. Finally, we demonstrated that ASC from all groups had an equal capacity to differentiate towards cardiomyocytes, as well as adipocytes and osteoblasts. In conclusion, we showed that significantly less stem cells were present in the SVF 1 day post-AMI; however, the stem cells that were present showed no functional differences. Fortunately, adipose tissue is a rich source of mesenchymal stem cells, still providing sufficient stem cells 1 day post-AMI for effective therapy.
Fetal Cardiac Extracellular Matrix Promotes Expansion of Neonatal c-kit+ Cardiac Cells In Vitro

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Methods to engineer cardiac tissue or expand cardiomyocytes in vitro for cell therapy face major limitations due to lack of proliferation of postnatal cardiomyocytes. The extracellular matrix (ECM) changes throughout heart development and maturation, and studies suggest it plays an important role in regulating myocyte proliferation. Here, we studied the effects of fetal, neonatal, and adult cardiac ECM on the expansion of neonatal rat atrial and ventricular cells in vitro. At 24 hr, cell attachment was lowest on fetal ECM; however most cells were cardiac alpha-actin+ indicating myocyte lineage, while many non-myocytes attached to neonatal and adult ECM and poly-L-lysine controls. After 5 days, the cardiac alpha-actin+ population remained highest on fetal ECM, with a 4 to 5-fold increase in number (Fig 1B). These cells often surrounded c-kit+ colonies, which were largest on fetal ECM (Fig 1A, C). Atrial cells formed larger colonies on fetal ECM and generally had more c-kit+ /cardiac alpha-actin+ cells compared to ventricular cells. Similar effects were not observed when cardiac cells were cultured on single ECM proteins (Collagen I, Collagen V, Fibronectin, Periostin), suggesting that the response to fetal ECM is not due to a single major ECM component. We are currently repeating these experiments using ckit+ progenitors derived from pediatric human patients undergoing surgeries for cardiac repair of congenital heart defects. Mimicking fetal cardiac ECM may be a novel approach for selectively expanding c-kit+/cardiac alpha-actin+ myocyte precursor cells for cardiac tissue engineering or cell therapy strategies.
Ultrasound Stimulation for Cardiomyogenesis from Embryonic Stem Cells

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Low intensity ultrasound (LIUS) is used for bone regeneration clinically. It has also been studied \textit{in vitro} to produce chondrogenic and osteogenic lineages from sources like stem cells. Considering the myocardial physiology, we propose that the mechanotransduction and mechanical stress that LIUS provide have great potential in cardiac regeneration. In this study, we investigated the effects of LIUS on cardiomyocyte differentiation of murine embryonic stem cells. After embryoid bodies were replated for 3 days, we treated the cells with 10 mins of ultrasound of different intensity and pulsing mode. Cells subjected to LIUS treatment generally produced higher expression of cardiac gene markers and higher beating rate than the controls. These differentiated cells also gave rise to cellular structure consisting of sarcomeric α-actin and cardiac troponin-T networks. The beneficial effects of LIUS were also demonstrated in 3-dimensional cultures of hydrogel-encapsulated embryonic stem cells. In conclusion, LIUS can be used to enhance cardiomyogenic regeneration for future cellular therapies of myocardial diseases.
The Functional Evaluation of ES cell-derived Cardiac Cell Sheet with Different Types of Fibroblasts

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Cell sheet-based tissue engineering technology enables to fabricate various types of tissue for transplantation and tissue models, it remains unclear that the suitable cellular network for cardiac cell sheets in terms of the fibroblast properties. In this study, we evaluated the function of cardiac cell sheets fabricated with mouse ES-derived cardiomyocytes and a variety of fibroblasts by assessing sheet forming ability, action potential propagation and cardiomyocyte proliferation. Mouse ES cell-derived cardiomyocytes were co-cultured with three types of fibroblasts (mouse neonatal cardiac Fibroblasts (NCFs), mouse adult cardiac fibroblasts (ACFs) and mouse adult dermal fibroblasts (ADFs)) on FBS-coated temperature responsive culture dishes for 5 days. In every condition, cell sheets were fabricated, but the time for When the extracellular action potential of each cell sheet was examined using multi-electrode system, action potential was observed in all over the area of the sheets with NCFs and ACFs, but not with ADFs. Immunocytochemical analysis revealed that cardiomyocytes in the cell sheets with NCFs and ACFs were elongated and evenly distributed, while those with ADFs showed the diminished size and localized. Furthermore, the number of cardiomyocytes in cell sheets with NCFs and ACFs was 2.5 times of that in cell sheets with NCFs. Now we are identifying the genes responsible for NCFs-mediated enhanced proliferation of cardiomyocytes. These findings suggest that fibroblasts derived from hearts might provide the appropriate environment for cardiomyocyte in the engineered cardiac tissue and the precise mechanisms of it remain to be determined.
Arterial bypassing, for small diameter vessels (<6 mm), suffers from poor patency rates using synthetic or autologous grafts due to thrombosis, aneurysm formation, and compliance mismatch. Elastin is a key component of native vasculature where it is responsible for the elastic recoil of vessels and has been shown to have a role in smooth muscle cell (SMC) behaviour. Thus, in this study, an elastin and collagen tubular composite scaffold was developed with the aim of providing a more natural viscoelastic response and to control SMC activity. An advanced biofabrication technique was developed which enabled the construction of a bilayered tubular construct via a combination of dehydration, freeze-drying and crosslinking. The resulting construct consisted of a highly porous outer layer for SMCs and a dense film layer to inhibit intimal hyperplasia and support endothelialisation. Results were evaluated via mechanical testing, histology, SEM, and biological response of seeded human SMCs. Results indicate that elastin caused a more natural viscoelastic response via increased creep resistance and improved recoil. The outer porous layer was found to have a pore size (~90μm) and porosity (~98%) in the ideal range to support cells while, interestingly, the addition of elastin was also found to stimulate cell proliferation. Spatially controlled crosslinking of the different layers was achievable due to the fabrication technique which allowed the ability to fine tune the mechanical properties of the layers to within the same range as native tissue. Ongoing research focuses on the application of in vivo-like cyclical strain and fluid shear stress within a custom-built vascular bioreactor to aid in the maturation of the engineered blood vessel.
Micropatterned Carbon Nanotube Embedded Cell-Laden Hydrogels for Cardiac Tissue Engineering

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Cardiovascular disease remains one of the leading cause of mortality in the worldwide with estimated 17 million deaths every year. Tissue engineering holds a great promise in replacement of damaged organs and tissue with biologically relevant engineered substitutes. Micro- and nanofabrication techniques have been proven to be powerful techniques to address the current challenges in tissue engineering. A major strategy in tissue engineering is the integration of innovative biomaterials with micro- and nanofabrication techniques to create constructs that recapitulate the in vivo cellular microenvironments. In this study, we used micropatterning technique and carbon nanotube (CNT) embedded gelatin methacrylate (GelMA) hydrogel to create highly organized cardiac tissue constructs. Our study included neonatal rat cardiomyocytes and cardiac fibroblasts as the primary cell types within the native myocardium tissue. Our results demonstrated that the engineered cardiac tissue constructs in CNT-GelMA hydrogels exhibited excellent mechanical and electrophysiological properties. Specifically, the constructs showed significantly higher spontaneous synchronous beating rates with lower excitation threshold compared to GelMA hydrogel. In addition, enhanced cell adhesion and cell-cell coupling were observed within the micropatterned features. Our findings indicated that CNT can be incorporated into hydrogel-based biomaterials to generate organized multi-functional cardiac tissue for in vitro studies and further therapeutic purposes. Furthermore, the micropatterned hybrid biomaterials could be used for other cell types to create tissue constructs with enhanced mechanical and electrophysiological properties.
The First Clinical Trial of Octacalcium Phosphate Collagen Composites in Dental Field

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Introduction: In preclinical studies, octacalcium phosphate and collagen composite (OCP/Col) demonstrated significant bone regeneration more than hydroxyapatite (HA) and β-tricalcium phosphate (β-TCP). The present study is the first clinical report to investigate the safety and the efficacy of this material when OCP/Col were implanted into defects after tooth extraction and cystectomy. The protocol of this clinical trial was approved by local institutional review board (IRB), and registered at International Clinical Trials Registry Platform Search Portal on World Health Organization. Ten adult patients signed the formal consent form and were included in the study. The disks of OCP/Col were implanted into the defects after 5 cases of tooth extraction and 5 cases of cystectomy. After implantation of OCP/Col, macroscopic, laboratory, and radiographic examinations were performed up to one year. After implantation of OCP/Col, postoperative course was stable and noticeable adverse event and laboratory disorders were not observed in all of the patients. In radiographic examination, radiopaque figure was chronologically enhanced in the OCP/Col implanted defect. Although OCP/Col itself had little radiopacity, the increase of radiopacity would be nucleated by the implanted OCP/Col. This study demonstrated the safety and efficacy of OCP/Col implantation after tooth extraction and cystectomy. OCP/Col would be a promising bone regenerative material in dental field.
Bone Marrow Mesenchymal Stem Cell Infusion in Intestinal Transplant Patients with Short Bowel Syndrome

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Small bowel transplantation is the most effective treatment for patients with short bowel syndrome and small bowel insufficiencies. We report enhanced epithelial chimerism after infusion of autologous bone marrow mesenchymal stem cells (BMSC) in patients undergoing cadaveric donor isolated intestinal transplantation (I-ITx). BMSCs were isolated from patients' bone marrow via iliac puncture and expanded in vitro prior to infusion. Here, we demonstrate a new strategy to administer BMSCs via the superior mesenteric artery of the graft enabling effective and direct access to the transplanted tissue. In this case report, we infused two out of three patients with autologous BMSCs and analyzed small intestine tissue biopsies collected post-operatively (8-69 months after surgery) for epithelial chimerism using XY fluorescent in situ hybridization (XY-FISH) and short tandem repeat polymerase chain reaction (STR-PCR). We observed significantly greater number of recipient type epithelial cells in the transplanted tissue with BMSC infusion in I-ITx compared to the case without BMSC infusion. Our findings suggest for the first time enhanced epithelial chimeric effect with autologous BMSC infusion in I-ITx. Potential implications of enhanced epithelial chimerism include improved graft survival, lower immunosuppressant doses, engraftment of the transplanted tissue, and as a consequence higher success rates in I-ITx.
Formation of stable articular cartilage is the ultimate goal and main challenge of cartilage tissue engineering. Since permanent articular cartilage is only generated during embryonic development, a better understanding of embryonic joint formation may provide essential clues for cartilage tissue engineering. Embryonic joint formation first becomes morphologically evident when the interzone forms at each prospective joint site. The interzone, consisting of 2 outer and an intermediate layer, constitutes a distinct cohort of progenitor cells responsible for the formation of the majority of joint tissues including articular cartilage. To date layer selective analysis of interzone cells has not been reported and therefore significant controversy exists regarding the exact role of the different interzone layers in joint development. In this study, microarray-based differential gene expression analysis of laser microdissected murine intermediate and outer interzone layers was performed. Pathway analysis revealed a high representation of inflammatory pathways and functions in the differential gene expression profile specifically in the intermediate layer. The 25 genes most highly differentially up-regulated in the intermediate interzone also have an emphasis on inflammatory pathways, while in the outer interzone they play roles in cartilage matrix formation, chondrocyte differentiation (hypertrophy) and endochondral ossification. Further studies are needed to look into the specific role of the inflammatory pathways in the interzone and to examine whether the differentially up-regulated genes relevant to chondrocyte hypertrophy and endochondral ossification in the outer interzone reflect its cell fate.
TissueGene-C (TG-C) in Patients with Osteoarthritis: A Placebo-Controlled Phase IIb Clinical Study

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TG-C is a cell mediated gene therapy that contains non-transduced (hChonJ) and transduced (hChonJb#7) human allogeneic chondrocytes. The hChonJb#7 cells were transduced with TGF-β1 gene by using retroviral vector and irradiated with gamma-ray. TG-C has been tested in Phase I and Phase IIa clinical studies in osteoarthritis (OA) patients and has proved its safety and efficacy in the patients. The current placebo controlled phase IIb study was conducted to determine both safety and efficacy of TG-C in patients with OA of the knee. Participants (n = 54) with a confirmed diagnosis of knee OA by X-ray and MRI were randomized into the treatment group (TG-C, 1.8x10⁷ cells/knee, n = 27) and the placebo group (saline, n = 27). The primary evaluation parameter was International Knee Documentation Committee (IKDC) which measures pain, sports activities, and daily function. The secondary evaluation parameters were Western-Ontario and MacMaster University (WOMAC) score, Knee Injury and Osteoarthritis Outcome Score (KOOS), and 100 mm Visual Analogue Scale (VAS). These parameters were assessed at 12 and 24 weeks post treatment. Additionally, changes in biomarkers were assessed in serum and urine samples. Safety measures, including physical exams, complete blood count, and serum chemistry were included up to 6 months post treatment. Blood samples were screened to detect the replication competent retrovirus (RCR), retrovirally transduced cells, and TGF-β1 DNA and protein starting from 2 weeks up to 6 months post treatment. TG-C treatment group showed improvement in IKDC, WOMAC, KOOS and 100 mm VAS scores compared to placebo group as shown in table 1.

Table 1. Changes in Scores of the Primary and the Secondary Evaluation Parameters

<table>
<thead>
<tr>
<th>at 24 weeks post treatment, ITT set</th>
<th>TG-C (N=27)</th>
<th>Placebo (N=27)</th>
<th>P values</th>
</tr>
</thead>
<tbody>
<tr>
<td>primary endpoint</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IKDC</td>
<td>8.05±11.18</td>
<td>16.39±15.63</td>
<td>P=0.0304</td>
</tr>
<tr>
<td>Secondary endpoint</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WOMAC</td>
<td>-7.50±13.02</td>
<td>-13.81±19.23</td>
<td>P=0.1692</td>
</tr>
<tr>
<td>KOOS</td>
<td>-10.79±18.22</td>
<td>-22.96±27.15</td>
<td>P=0.1508</td>
</tr>
<tr>
<td>VAS</td>
<td>-10.79±18.22</td>
<td>-24.83±27.29</td>
<td>P=0.0321</td>
</tr>
</tbody>
</table>
Articular cartilage defects have no regenerative capacity. Although various tissue engineering strategies are available, none of them provides optimal results. An innovative new strategy to treat articular cartilage lesions is the implantation of autologous chondrocytic spheroids (Co.don, Teltow, Germany). We present the first histological re-evaluation of second-look biopsies to analyse the regenerative effects of this treatment. Standardized synthesized autologous chondrocytic spheroids were implanted in articular cartilage lesions of four patients. Second-look biopsies were taken between four and sixteen months after implantation during a second arthroscopy, which was indicated independent of the spheroid-based autologous chondrocyte transplantation (ACT). Biopsies were fixed in buffered formalin and prepared according to standard methods for conventional histological (HE, alcian-blue) and immunohistochemical (collagen II, aggrecan, lubricin) examination followed by semiquantitative evaluation. Second-look biopsies showed typical articular cartilage architecture consisting of flat cells in the superficial zone, and round shaped chondrocytes producing large amounts of alcian-blue positive extracellular matrix components. Fibrous tissue or vascularization was not detectable. Immunohistochemicaly the superficial cell-layer was lubricin-positive, and collagen II and aggrecan were detected in large amounts. To our knowledge this study is the first histomorphological evaluation of scaffold-free spheroid-based ACT in humans. The impact of spheroid use for the regeneration of hyaline cartilage in articular lesions is clearly demonstrated.
Combined use of transalveolar distraction with biomimetic materials in construction of large size alveolar defects in unilateral complete cleft lip and palate; (A controlled clinical trial)

Hassan Mousssa, Ahmed Medra, Yasser Helmy and Mona Marei

To compare and analyze the effect of different biomimetic materials in construction of crater like defects resulting after transalveolar distraction during treatment of large dentoalveolar defects in a group of patients presenting with unilateral complete cleft lip and palate. 35 patients, 13-18 years with UCLP manifesting large alveolar defects minimally 3 teeth absent including its alveolar housing, were randomly selected from patients presenting to cleft lip and palate center Faculty of Dentistry Alex-Univ. Patients were randomly divided into 5 groups according to the type of grafting materials used to construct the residual defect following transalveolar distraction into:

- Group 1: Autogenous bone graft (control group)
- Group 2: Autogenous bone with resorbable membrane
- Group 3: Freeze dried bone with resorbable membrane
- Group 4: Tricalcium phosphate 30% +70 % Hydroxy apatite with resorbable membrane
- Group 5: Hydroxy apatite (1000-600 microns+600-250 microns) with Platelet rich fibrin.

Cone beam CT was done in the following manner for all groups:

- T0-Pre treatment
- T1-After orthodontic expansion 4-6 mm
- T2-After TAD (1 month of distraction)
- T3-After reconstruction (6 months after grafting)

1- All 35 cases showed complete closure of cleft site without fistulae
2- Size of defect that was closed had a mean value of 3.9ccm$^3$ ± 0.8 ccm$^3$
3- Bone formation after grafting was evaluated by densitometry, showed similar results in group 2 and 5 the most superior compared to control group. Group 3 and 4 showed complete bone formation yet its quality was less superior to control group.
4- Soft tissue healing for group 5 was most superior to any of the 4 other groups, using probing depth, time required to remove surgical stitches and bleeding tendency.

TAD with biomimetic material is a valid method for treatment of severe alveolar defect and PRF shows best soft tissue healing response.
MRI Demonstrates Improvement in Cartilage Following Intra-articular TG-C Treatment; Case Report

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\textsuperscript{1} Qmetrics Technologies, \textsuperscript{2}Kolon Life Science, Inc., \textsuperscript{3}TissueGene Inc.

A randomized single-blind phase IIa trial was conducted in 28 patients with knee OA to determine both safety and efficacy of TG-C. TG-C is a cell mediated gene therapy that contains non-transduced (hChonJ) and transduced (hChonJb#7) human allogeneic chondrocytes. hChonJb#7 were transduced with TGF-β1 gene using retroviral vector, while hChonJ were not modified. The hChonJb#7 cells were then irradiated with gamma-ray for preventing any chance of DNA integration of the retroviral vector to human genome. MR images acquired at Baseline (pre-treatment), as well as 6 and 12 months post-treatment, were evaluated by two experienced musculoskeletal radiologists in a blinded fashion using a modified WORMS scoring method. After this scoring, the images were presented to the two Radiologist again, in time sequence, and radiological impressions were generated for cartilage, and any significant visible trends or changes were identified. During the un-blinded radiological evaluation a set of patients was noted to have improvements in the cartilage. The following improvements were observed: full thickness cartilage defect filling, generalized cartilage thickening in the defect area, and improvement or resolution of cartilage blisters. One patient showed several of these findings, and additionally showed considerable bone regeneration (bone remodeling) and cartilage regeneration in the trochlea, restoring the trochlea shape. Although these patients demonstrated many areas of improvement, in some patients there was evidence of progression of OA as well. Based on these findings, there is an indication that in certain cases this treatment induced cartilage regeneration.
Bone Formation of Degradable Silk Fibron Composite Materials

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Silk materials is made by silkworm intensively since 3days 5 instar. Silk is known one of a good biocompatible materials because silk thread has been used as a surgical suture until now. Silk fiber is strong and tough fiber and is good chemico-physical resistance to degradation. Silk is aproveved as a non-degeradable boimaterials by FDA. Silk fibron was dissolved and cast on the casting board with a anti-microbial agent. The silk membrane examined for GBR technique. The rabbit tibia model was used for in vivo test. New bone formation was evaluated by removal torque test and histomorphometrical evaluation. Silk materials could successfully regenerate bone in the rabbit tibia peri-implant bone defect model. Therefore, Silk materials with incorporated with a anti-microbial agent will be useful for GBR technique in many types of bone defect.
Tooth loss or partial damage of tooth structure affects people adversely. Dental pulp removal caused by irreversible damage makes the whole tooth lose vitality and defensive ability, finally resulting in tooth extraction. Although there are many ways to make a substitute, a tissue-engineered dentin-pulp-like structure would provide a vital alternative to currently available clinical treatments. To meet such purpose, mesenchymal cells were isolated from newborn porcine molar dental germ and sub-cultured in vitro. Sodium hyaluronate (HA) gel, as an injectable scaffold, was prepared by means of cross-linking using 1,4-butanediol diglycidyl ether as a crosslinker. The sub-cultured cells were then combined with HA gel at a concentration of $5 \times 10^7$ cells/ml. TGF-β1 was further supplemented within the above composite at predetermined concentrations. The final composite was then injected subcutaneously in nude mice. After 10 weeks, a recognizable dentin-pulp-like structure was formed which was composed of odontoblasts, predentin and dentin according to H&E staining. Furthermore, dentin sialoprotein (DSP) and typical dentinal tubule structure can be identified by immunohistochemical staining. Based on our results, it was demonstrated that, the injectable HA gel is a suitable template for scaffolding mesenchymal cells originated from tooth germ, resulting in regeneration of dental pulp tissue with minimal invasive surgery. The current work further shed light on the clinical application of on-site regeneration of bioengineered partial dental tissue.
Towards a Novel Tooth In situ Organ Culture Model for Periodontal Research

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* Contributed equally to this work and are noted as joint authors

Periodontal disease (PD), resulting in the progressive loss of the tooth attachment, is the second most common disease in the western world. The clinical challenge of treating chronic PD has resulted in increasing interest in identifying ways to regenerate the tooth-supporting tissues. However, current in vitro models lack appropriate physiological simulation and do not comprise of the whole tooth in situ functional unit. The aim of this study is to develop a porcine tooth in situ organ culture model suitable for studies of periodontal regeneration, ultimately under appropriate physiological loading in health and disease. A total of 18 porcine first molars were dissected aseptically in situ within their bony sockets. Twelve were used to optimise sterility while maintaining tissue viability. Six were used in a 4 day organ culture study. Periodontal pocket depths were measured for each tooth before culture in medium with antibiotics. Cell viability was determined at days 1 and 4 using XTT assay. Samples obtained from the culture medium and the sub gingival, gingival, intra radicular and surrounding bone were assessed using microbiological culture (aerobic and anaerobic), XTT assays indicated viability throughout the culture period for all tissues investigated (gingival, periodontal ligament, alveolar and cortical bone). Microbiological cultures were clear confirming maintenance of sterility within the organ culture system. In conclusion, a whole tooth in situ organ culture system was successful over four days maintaining both sterility and tissue viability. Currently the organ culture model is being tested within a novel bioreactor to simulate physiological loading for future periodontal research.
Novel Remineralising, Antimicrobial Dental & Orthopaedic Resin-Based Composites

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Novel resin-based composites with added tissue-remineralising and antimicrobial properties are being developed for tooth restoration and bone repair. Problems with current composites include poor strength and biocompatibility resulting from low polymerisation upon setting (<50%) and subsequent release of small diluent monomer molecules. Furthermore, the high double bond density of these small monomers gives rise to high shrinkage and heat generation during the setting reaction, which may lead to tooth de-bonding or surrounding bone tissue damage respectively. Other problems include limited release of antimicrobial agents (<5%) of that included) and low toughness. In this study therefore, composites were produced using novel diluent monomer PPGDMA. Its high flexibility and molecular weight enabled greater monomer conversion (>70%) without an increase in composite shrinkage. Furthermore, use of a polymerisable and adhesive co-initiator enabled improvement of bonding to collagen in demineralised dentin. Subsequent addition of reactive calcium phosphates promoted rapid formation of hydroxyapatite on the set material surfaces in simulated body fluid but not artificial saliva. This could enable dentine and bone remineralisation without roughening of the aesthetic surface of tooth restorations. Over 60% of an added antimicrobial agent was released from these composites over a six-week period. The materials also had comparable or greater strength and significantly improved toughness compared with current market-leading composites for both tooth and bone repair.
Treated With Hydrofluoric Acid Repair Using Cryogel Scaffolds Loaded with BMP-7-Expressing Primary Chondrocytes

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The aim of the study was to investigate solely the effect of fluoride on titanium dioxide (Ti) on the surface chemistry and its effect on proliferation and differentiation of primary human osteoblasts (NHO).

NHO cells were exposed to fluoride-modified and unmodified samples for 1, 3, 7, 14 and 21d. The fluoride effect on the mRNA expression was quantified and measured. The secretion of cytokines and interleukins in the cell culture medium was measured by Luminex, gene expression by RT-PCR, and compared to untreated controls. The effect on cell growth after 1- and 3d in culture was measured using [³H]-thymidine incorporation.

No increase of cell proliferation was found amongst fluoride modified Ti surfaces compared to controls. The cell differentiation regarding to gene expression showed no significant differences in both fluoride modified and unmodified samples, and less effect on protein release.

The fluoride from hydrofluoric acid treatment on Ti surfaces gave no specific effect on primary human osteoblast cells.
Assessment of Mesenchymal Cells Isolated from Tooth Pulp and Composite Scaffolds Produced by Surface Selective Laser Sintering as Components of Bone Tissue Engineering Constructs

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We are trying to develop a tissue engineering approach to the reconstruction of bone defects using dental pulp-derived mesenchymal stem cells (MSCs) seeded onto polymer or polymer-mineral scaffolds fabricated from polylactoglycolide with addition of bioactive calcium phosphate ceramics by surface selective laser sintering (SSLS). Cultures of mesenchymal cells were derived from the pulp of human deciduous teeth. Presence of MSCs in the culture was verified by cell surface marker expression profile (CD29+, CD34-, CD44+, CD45-, CD49b+, CD73+, CD90+, и HLA-DR-). Experiments with various materials (brushite-hydroxyapatite cement, hydroxyapatite cement, tricalcium phosphate prepared by the conversion of gypsum, tricalcium phosphate ceramics) were carried out to select those allowing better proliferation and efficient osteogenic differentiation of the cells. Of the materials tested, a greater degree of cell survival and proliferation was observed in case of culturing the cells in the presence of tricalcium phosphate ceramics (Fig. 1, A). Tricalcium phosphate ceramics visibly enhanced osteogenic differentiation as evidenced by the increased number of calcified nodules and increased extracellular matrix production (Fig. 1, B). Based on these results we have chosen to incorporate tricalcium phosphate ceramics into polylactoglycolide scaffolds with SSLS. The scaffolds were then successfully seeded with MSCs (Fig. 1, C). Cells cultured with this material remained viable for a long time, thus providing data confirming the possibility of clinical usage of our technology in the field of bone regeneration.
Silver Deposition on Titanium Surface by Electrochemical Anodizing Process Reduces Bacterial Adhesion and Growth of Streptococcus Sanguinis and Lactobacillus Salivarius

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Titanium dental implants sometimes fail due to factors like lack of osseointegration or peri-implantitis. Peri-implantitis describes inflammatory reactions with loss of supporting bone around an implant due to bacterial infection leading to its failure. To avoid it is important to maintain dental implant surfaces free of bacteria. This study determines the antibacterial properties of silver doped titanium samples prepared with a novel electrochemical anodizing process. Incorporation of silver on titanium by anodizing presents clear advantages for its application because anodizing is a common manufacturing process for dental implants. It increases the width of the passive titanium oxide layer acting as a barrier against metallic ion release, reduces surface reactivity and increases corrosion resistance in the physiological medium. Titanium discs were anodized in an aqueous solution of silver nitrate (AgNO3) and sodium thiosulphate (Na2S2O3) at room temperature with agitation. SEM, white-light interferometry, contact angle and XPS were used to determine physico-chemical surface properties. In vitro viability of treated samples was studied with Hffβ fibroblastic cells in a LDH assay. Antibacterial properties were tested against S. sanguinis and L. salivarius by adhesion bacterial and growth curve assay. XPS confirmed the presence of silver on treated titanium samples, seen as localized deposits in SEM imaging. A higher concentration of AgNO3 and the use of iterations increased the silver deposition on titanium surface. No cytotoxicity was detected in the LDH assay. A significantly reduction on bacterial adhesion and growth was achieved with samples anodized for 500 iterations on a high concentration electrolyte.
Healthy mucosal seal around the dental abutment is an important factor for long term success of implants. Compared to studies on improving bone healing, there is a lack of investigations regarding integration of abutment within soft tissue. Here we employed cathodic polarization to modify titanium surfaces. Three different organic electrolytes were used: oxalic acid, tartaric acid and acetic acid. Reaction durations varied from 0.5 h to 5 h and current density from 1 mA/cm$^2$ to 15 mA/cm$^2$. Surface topography was analyzed by blue light profilometer, field emission scanning electron microscopy and atomic force microscopy. Secondary ion mass spectrometry was used to quantify elemental compositions on the surfaces. Hydrophilicity was determined by contact angle measurement. Samples were evaluated in vitro performances using human gingival fibroblasts. Surface topography and chemistry were affected by the electrolyte composition, the current density and the polarization time. Under identical conditions, oxalic acid created rougher surfaces than tartaric acid and acetic acid, while acetic acid produced the most surface hydride. The amount of surface hydride was positively correlated with polarization time but not to current density. Proliferation rate of human gingival fibroblasts at day 3 was positively correlated with the surface hydride content. The improved performance was suggested to be due to increased hydride content, rather than changes of surface complexity or hydrophilicity.
New Sol-Gel Coatings for Dental Implants: Biological Evaluation

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Due to its biocompatibility, resistance and osseointegration capability titanium (Ti) is the most widely metal used to manufacture dental implants. The required time to achieve the incorporation of a dental implant in the bone tissue of a human patient is usually between 4 and 6 months, and this time is longer in metabolic disease patients. So, the addition of osseoinductive properties to the chemical implant surface abbreviate the time of bone tissue repair. Our research team have developed organic-inorganic coatings that release Silicon (Si) by its hydrolytic degradation, providing the titanium implant surface with osseoinductive behaviour. In this work, the induction that new hybrid coatings have on cell proliferation and calcium deposits formation was measured in vitro. The biocompatibility, osseointegration and osseoinductive properties of the coatings were evaluated through in vivo implantation in rabbit tibia. The results of biocompatibility and osseointegration capabilities were evaluated via histology and the osseoinduction capability was evaluated via EDX microanalysis. Sol-gel coatings induced more bone matrix formation than Ti by means of Si release that increases with in vivo material degradation rate. The new organic-inorganic coatings developed in this work are biodegradable, biocompatible and osseoinductors, presenting valuable characteristics for their application on Ti dental implants treatments and to enhance the total implant osseointegration.
The potential of dental stem cells in regenerative medicine

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The ability to control the behaviour of stem cells, which is regulated by their environment, is an essential goal in the field of regenerative medicine. Since the discovery of dental mesenchymal stem cells (DMSCs) in human teeth their potential for the use in cell-based therapies has been studied intensely. Understanding the migration, proliferation, and differentiation capabilities of various DMSC populations in artificial or natural 3D environments will support the development of new therapeutic approaches for tissue repair. Human dental pulp and dental follicle stem cells (DPSCs/DFSCs) were grown in mono- and co-cultures. Migration and proliferation were analysed by time-lapse imaging and regulatory genes involved in dental cell migration and differentiation were measured by qRT-PCR. DPSCs and DFSCs presented low and irregular migration profiles under mono-culture conditions. In co-cultures, DFSCs showed an increased migration activity and velocity and surrounded the DPSCs. Gene expression profiles of DPSCs and DFSCs were also influenced. Furthermore, DPSCs seeded on 3D silk scaffolds gave rise to mineralized structures, which were increased in the samples grown in spinner flask bioreactors in the presence of osteogenic medium by a factor of 10.5±4.7 when compared to the static control grown in standard culture medium.

The present findings prove that the environment does influence the behaviour of DPSCs and DFSCs, which keep their genetic memory and compete with each other for territory \textit{in vitro}. Mechanical loading was shown to play an important role in the mineralization process. Future studies will investigate the effect of dentinogenic factors on the behaviour of DMSCs.
Development of an Injectable bi-Layered System for Platelet Lysates Sustained Delivery in Support of Periodontal Tissue Regeneration

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Periodontitis is a severe infection responsible for the destruction of the tooth supportive tissues, including the periodontal ligament (PDL) and alveolar bone (AB). An effective strategy for reconstruction of periodontal damaged tissue is yet to be achieved.

This study proposes the development of a bilayered system for the regeneration of AB and PDL, consisting of two parts: a calcium phosphate (CaP) cement incorporating hyaluronan (HA) microspheres produced by a spray-drying method and loaded with Platelet Lysates (PL), aiming for AB regeneration; and a PL-based hydrogel for PDL and cementoblast layer restoration. PL is a source of multiple growth factors which can stimulate the recruitment and osteogenic differentiation of stem cells. The HA microspheres loaded with PL incorporated on the CaP cement can improve its degradability, acting simultaneously as a controlled delivery system of bioactive factors. Micro-computed tomography of the scaffolds showed uniform distribution of the microspheres throughout the cements. The incorporation of HA microspheres largely increases the loading ability and the sustained release of proteins in the constructs. Moreover, the sustained release of PL from CaP cements enhances the expression of early osteogenic markers in human adipose derived stem cells seeded onto the constructs, suggesting the potential of the proposed system for AB regeneration applications. New injectable and stable PL-based hydrogels were also designed to support ligament tissue regeneration, which, in combination with the above described cements, shall enable the full regeneration of periodontal defects. The proposed model is an appealing strategy for the regeneration of the periodontium as demonstrated by ongoing trials in rat models.
Local Gentamicin Application Does Not Interfere with Bone Healing in a Rat Model

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Since several decades antibiotics mixed with bone cement are successfully used in prosthetic surgery and a gentamicin coated tibial nail is approved for fracture stabilization in Europe. The goal of the present study was to investigate if gentamicin, locally applied from a polymeric coating of intramedullary nails, might interfere with the bone healing process. Rat tibiae (n=72) were osteotomied in an open approach and intramedullary stabilized with a nail. This model shows a delayed healing with a prolonged inflammatory reaction. The open approach is clinically relevant because it mimics the clinically critical case of an open fracture, which has a higher risk of infection. The nail was either coated with the polymer only or with 10% gentamicin incorporated into the coating. In vivo µCT analyses were performed over the healing period. Mechanical testing & histological evaluation were done at the days of sacrifice: days 28, 42, and 84. µCT analysis revealed an increase in tissue mineral density (TMD) over the healing period in both groups. The torsional stiffness and maximum load reached in the control group at no time point the values of the intact contra lateral side. At day 84 the gentamicin treated tibiae, however, showed significantly better maximum load compared to the control group. The histology showed no bony bridging in the control and 3 of 5 calluses of the gentamicin group still contained cartilage. Significantly more mineralized tissue was measured in the gentamicin group. This study shows that the local gentamicin application does not negatively interfere with the healing process. Both groups showed healing progression with an increase in mineralization, whereas the gentamicin treated calluses showed a slightly improved healing.
Pharmacologically active microcarriers (PAMs) with a laminin biomimetic surface, releasing neurotrophin-3 (NT-3) combined to marrow-isolated adult multilineage-inducible (MIAMI) cells showed interesting potential for Parkinson’s disease (PD) therapy (Delcroix et al., 2011). We further investigated the mechanisms underlying tissue-regenerative effect of MIAMI cells and Neural Stem Cells (NSC) combined to PAMs. We hypothesize that NSCs will replace the lost dopaminergic cells while MIAMI cells will protect the nigrostriatal pathway by secreting tissue-repair factors. We developed an organotypic model of PD enabling an easy, rapid and reproducible investigation tool. Stem cells alone or in combination with laminin-coated PAMs releasing NT-3 were injected into the striatum of the slices and their behavior was analyzed at 1 day and 2 weeks after implantation. Both implanted stem cells differentiated into neuron-like cells expressing β3-tubulin, tyrosine hydroxylase and were able to secrete dopamine. Dopaminergic differentiation was better with NSCs and enhanced by the PAMs releasing NT-3. Repair of the nigrostriatal pathway was observed 2 weeks after injection of the stem cells, particularly with MIAMI cells complexed to PAMs releasing NT-3, probably due to the increased survival and secretion of growth factors. Secreted vascular epithelial growth factor might be responsible of their neurorepair effect, directly or indirectly through the protection of the microvasculature around the graft, which was enhanced after implantation of MIAMI cells/PAM complexes.

In conclusion, these results show the potential interest of this approach for tissue engineering strategies.
Drug-Loaded Biofunctionalized Carbon Nanotubes for Neural Regeneration

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Carbon nanotubes (CNTs) take great interests in biomedical applications to design new innovative biomaterials for nerve regeneration. Modification with biologically-relevant and effective molecules on CNTs in terms of biofunctionalization has been of great importance to improve functionality in neural regeneration. In this study, we used a new method to coat the cell culture surfaces by covalently bonding with biofunctionalized multi-walled carbon nanotubes (MWCNTs) and to load drugs, followed by characterization, biocompatibility and neuronal outgrowth analysis of CNT substrates on PC12 cell lines. Our results demonstrate that biofunctionalized CNTs can make it possible to incorporate drugs for long-term sustained release and serve as effective substrates for neuronal growth. We also show that bpV-loaded biofunctionalized MWCNT substrates can down-regulate the expression of PTEN proteins and up-regulate AKT/ERK signaling pathway, which might be the underlying mechanism for the improved differentiation in PC12 cells for guiding the neurite outgrowth. These apparent results suggest our conductive biomaterials based on drug-loaded biofunctionalized CNT substrates can provide better surfaces and enable enhanced neural adhesion and promoted neurite extension through its electrical stimulation, nano-scale topographical environments, and controlled release drug delivery. This may provide insight for future therapeutic application of CNTs in neural regeneration.
Injectable Hyaluronic Acid-liposome Hybrid Hydrogel for Multi-drug Delivery

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Multi-delivery systems that could carry various molecules and release them systemically are normally more efficiency for the tissue regeneration1 and drug therapy2. In this study, we have developed an injectable liposome-hybrid hyaluronic acid (HA) hydrogel for multidrug delivery. This system could be used for targeting cancerous tissues since HA, a naturally polysaccharide with no immunogenic effects, could interact specifically with cancer cells via its major receptor CD443.

Rhodamine-labeled liposomes that functionalized with carbonate group were prepared using a thin-film method. Meanwhile, HA polymers were functionalized with hydrazide or aldehyde groups for the hydrogel formation. The modified liposomes can be either linked to the HA hydrogel matrix or coated with HA polymer via a “click” reaction with aldehyde-modified HA. Systemic release can be achieved by first releasing the molecules in hydrogel matrix due to diffusion and/or hydrogel degradation (by enzyme hyaluronidase (Hase)), and then the molecules inside the liposome will be released later on due to the cellular uptake of liposomes.

Lipsomes were successfully prepared confirmed from TEM. According to the release, calcein loaded inside HA matrix released within 2 h no matter there was Hase or not due to the too low molecular weight of calcein (623 Da). In contrast, there was no leakage of calcein from liposomes under physiological condition (PBS buffer without Hase) which means the membranes of this type of liposomes are quite compact so that the loaded compound could not diffuse out. In the presence of Hase, liposomes could release slower from the hydrogel due to the enzymatic degradation.

By taking advantage of “click” chemistry, we could design the delivery system for multi-compounds. On the other hand, since the liposomes are covalently linked to HA matrix and their membranes are so compact that could keep the dye inside, this type of liposomes could also be used as probes for labeling the hydrogel.

Fig. 1. **Left**: overview of the formation of HA-liposome hybrid hydrogel. **Right**: *In vitro* release of calcein from HA hydrogel and HA-liposome hybrid gel with/without hyaluronidase (Hase)
Incorporation of Degradable and Integrin Binding Peptide Sequences into Poly (ethylene glycol) Hydrogel Coating of Magnetic Iron Oxide Nanoparticles for Targeted Delivery into Tumor Cells

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Specific targeting of tumor cells is one of the main limitations in most drug based therapies including cancer treatment. However, side effects and resistance of cancer drugs prevent efficient therapy. These challenges could be addressed through specific drug targeting and controlled drug release strategies, which will allow for high accumulation of drug in target tissues. Recently, we have demonstrated that RGDS-functionalized poly (ethylene glycol) (PEG) hydrogel coating around magnetic iron oxide nanoparticles (MIONPs) significantly enhance accumulation and specific cellular uptake into cancer cells. We have extended this work to include collagenase sensitive peptide sequence within PEG hydrogel coating. Covalently bonded RGDS sequence within functional PEG hydrogel enhances specific internalization of the nanoparticles into cancer cells, and maintain accumulation of nanoparticles around cancer tissue, where covalently bonded degradable sequence within PEG hydrogel scaffold allows for the release of therapeutic molecules within the coating towards their surrounding as a result of high expression of metalloproteases by cancer cells. This multifunctional property of coating is promising for efficient and targeted therapy without undesirable side effects. The approach developed here may also be useful for simultaneous diagnostic and therapeutic imaging of cancer tissues and for targeted drug delivery into the tumor site.

Keywords: Specific targeting; enzymatically degradable PEG hydrogel; magnetic iron oxide nanoparticles.
Non-viral polymeric carriers offer high potential as an alternative means of drug delivery. Bone-seeking osteotropic materials like Hyaluronic acid (HA), form an attractive option for targeted therapy. In this study, chemically modified HA was linked with a bisphosphonate moiety and used as a polymeric carrier to deliver the drug in vitro to osteoclast-like cells. In order to visually follow the fate of the carrier, the polymer was additionally functionalized with FITC as a fluorophore. The murine macrophage cell line RAW 264.7 cells was differentiated into osteoclast-like cells with addition of RANKL. Osteoclast formation was confirmed with TRAP assay and nuclear staining. The differentiated cells were incubated with defined concentrations of the HA-linked bisphosphonates with relevant controls. Confocal microscopy was performed to localize the carrier inside the cells and cytotoxicity studies were performed to evaluate the toxicity of the drug-linked polymer and free carrier. Results indicate that the bisphosphonate linked polymer has a higher potent effect compared to the bisphosphonate alone, whereas the HA itself has limited potency against osteoclasts. The polymer-linked drug does not seem to have any inhibitive effect against osteoblasts in vitro. (Functionalization and imaging of biomaterials (MultiTERM))
Novel Hydrogels for Site Specific Delivery of Human Mesenchymal Stem Cells and MicroRNA Mimetics as a Multimodal Therapeutic for the Treatment of Myocardial Infarction

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The European Society of Cardiology estimates that every sixth man and every seventh woman in Europe will die as a result of a myocardial infarction (MI). Current therapies are ineffective in restoring full cardiac function post-MI. Mesenchymal stem cells (MSCs) have gained attention as a restorative treatment option due to their ability to promote myocardial regeneration but their utility is limited by low levels of cell engraftment following delivery. This project aims to incorporate MSCs into a thermoresponsive hydrogel matrix in combination with pro-survival/angiogenic microRNAs (miRNA) to increase the levels of MSC engraftment at the site of infarction. MSCs were seeded within thermoresponsive chitosan/β-Glycerophosphate gels. Cells were Live/Dead stained at multiple time points to assess viability within the thermoresponsive chitosan gel. Assessment of dsDNA levels were also carried out to validate the ability of the cells to proliferate within the chitosan gel. Fluorescently tagged miRNAs were loaded into a thermoresponsive pegylated tyrosine gel and miRNA release into PBS was quantified by fluorescence spectroscopy over multiple timepoints. Results indicate that the chitosan gel is suitable for MSCs delivery as the cells were viable and proliferated over the five day period. miRNA release within the pegylated tyrosine gel showed a sustained continuous release profile over a five day period which is favoured over simple bolus delivery. The next step is incorporation of these dual modalities within a bioactivity model to assess angiogenic potential.
Polymer networks, hydrogels, have been used in many biomedical applications such as materials for the tissue engineering and drug delivery. Controllable drug release is one of the advantages of using the hydrogel-based drug delivery systems in biomedical applications. However several unresolved issues have impeded the wider therapeutic hydrogel applications. One of them is the quantity and homogeneity of hydrophobic drug loading into hydrogel is limited by poor solubility of the drugs in aqueous solution. In this work novel polymeric gels with affinity to both water and organic liquids have been synthesised. The gels were prepared by co-polymerisation of 2-acrylamido-2-methyl-1-propane sulfonic acid (AMPS) with acrylamide (AAm) and N,N′-methylene-bis-acrylamide (MBAm). AMPS was neutralised by triethylamine (TEA) before polymerisation. Poly(AMPS-AAm) gels exhibited superabsorbancy in both water and series of alcohols. The hydrophobic drug, chloramphenicol, uploading into the gels was much greater in alcohol than that achieved in water. The poly(AMPS-AAm) gels exhibited a similar drug release profile with conventional hydrogels; and only part of the drug uptaken by the gels was released as a result of poor aqueous solubility of chloramphenicol.

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Auricular Cartilage Repair Using Cryogel Scaffolds Combined Delivery of Osteogenic Agents by Polymeric Micelles

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Stem cells, isolated from dental tissues (i.e. human periodontal ligament mesenchymal stem cells, hPDLSC) can offer new therapeutic opportunities to patients suffering from tooth loss or periodontal destruction. The identification of factors promoting osteogenesis in hPDLSCs is critical for their potential in in vivo regeneration of bone tissue. New alternative strategies to induce osteogenic differentiation and to optimize the drug delivery to cell have been analyzed. In this context, the use of polymeric micelles (PMs) for the differentiation induction could result in a series of advantages including simultaneous controlled delivery, increase of drug availability, cellular distribution and drug uptake by the target cells. The current work reports the production of PMs for the co-delivery of dexamethasone (DEX) and ascorbyl-palmitate (AP) to in-vitro cultured hPDLSC for the combined induction of osteogenic differentiation. PMs, based on pluronic block-copolymers, were produced with a microfluidic platform to achieve a control on the PM size. Our results demonstrated that drugs were co-entrapped in PMs and that different production parameters can be adjusted in order to modulate the PM characteristics. The comparative analysis of PMs produced by microfluidic and conventional procedures confirmed that microfluidics allowed the production of PMs in a robust and reproducible manner. Finally, the analysis of the effect of PMs, containing DEX and AP, on the osteogenic differentiation of hPDLSC demonstrated the effectiveness and safety of treatment. In conclusion, the microfluidic approach represents an innovative method for the controlled preparation of nanotechnology based formulations for regenerative medicine.
Preparation and Characterization of Biodegradable Composite Systems as Hard Tissue Supports: Bone Fillers, Bone Regeneration Membranes and Scaffolds

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In tissue engineering applications, poly(ε-caprolactone) (PCL) is one of the preferable polymers used in the restoration of the bone defects due to its desirable mechanical properties and biocompatibility. Addition of inorganic calcium containing particles (either as calcium phosphate or hydroxyapatite) can improve the mechanical properties as well as osteoconductivity. In this study, biodegradable hard tissue supports were prepared as particulate fillers, 2D membranes and 3D scaffolds. Beta-tricalcium phosphate (β-TCP) was synthesized and microspheres were prepared from the synthesized β-TCP via gelatin mixing and were loaded with gentamicin. Also films and porous scaffolds were prepared by adding these microspheres into polycaprolactone (PCL) matrix. In vitro gentamicin release studies indicated β-TCP/Gelatin composite fillers have effective sustained release and less burst release compared to drug loaded pure β-TCP powder. Both the burst release amounts and sustained release periods increased with increasing the addition of fillers. The morphology of the partially degraded samples of composites showed that, fillers have effect on the constitution of the fibrous structure which can enhance the cell attachment and proliferation in the matrix. On the other hand, antibacterial activities of all gentamicin loaded systems against microorganisms of E.Coli and S.Aureus were observed.
Thermoresponsive Release of a Pro-Osteogenic Peptide from a Collagen-Hydroxyapatite Scaffold for Bone Tissue Engineering

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In the recent years, significant advances have been made in the development of scaffolds which mimic the extracellular matrix for bone tissue engineering (TE). Nevertheless, the effective and safe release of biomolecules which enhance bone tissue healing from these scaffolds, essential for the regeneration of large fractures, still remains a challenge. Ideally, a scaffold for bone TE should replicate the naturally occurring complex cascade of growth factors for achieving an enhanced regeneration. One way for enabling this would be the development of a responsive scaffold which delivers pro-osteogenic factors under external stimuli. In the present work we describe, for the first time, a thermoresponsive pro-osteogenic peptide eluting collagen- hydroxyapatite (CHA) scaffold designed specifically for bone repair. Peptide loaded biocompatible thermoresponsive liposomes were attached to the chemically modified surface of highly porous CHA scaffolds. The released model molecule is a pentapeptide of the Parathyroid Hormone related Protein (PTHrP 107-111), which has shown pro-osteogetic and antiosteoclastic properties. The extensive characterization carried out in this study, including confocal microscopy, mechanical testing and peptide delivery studies, shows that the functionalization process do not alter the properties of the scaffolds and that the attached liposomes are homogenously distributed through the structure. It has been also shown that release kinetics of the peptide can be effectively controlled by external heat stimuli. These novel results confirm that these liposomes-scaffolds constructs can be the basis for the development of remotely-controlled sequential multifactor release devices.

Keywords: Bone, collagen-hydroxyapatite scaffold, peptide delivery, functionalization, liposomes.
New Method for the Perioperative Enrichment of DBM with Antibiotics

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Bony infection cause dramatic discomfort for the patient, resulting in healing impairment, reoperations, and increased health care costs. Infection prophylaxis and therapy by using antibiotic loaded bone grafts have the advantage of a high local concentration while antibiotic dosage and systemic side effects are reduced. Cements loaded with antibiotics are used in endoprosthetic surgery, however, they do not support bone regeneration. For the optimal treatment, an individual mixing of a specific antibiotic with a grafting material supporting bone healing is important. The present study aims in the investigation of an easy and reliable perioperative mixing procedure of different antibiotics with a demineralized bone matrix (DBM). Both preparations of antibiotics, powder or solution, can easily, homogeneously and reproducible be mixed with the graft using a special syringe. The used antibiotics show different release profiles: complete release of Gentamicin and Tobramycin within 3 days, complete release of Vancomycin within 14 days. Inhibition of bacterial growth (Staph. aureus) was detectable for at least seven days. Osteoblastic cell viability and alkaline phosphate activity was reduced only with the high concentrations of the antibiotic released at the early elution time points (1h & 1 day). The later elution samples had no negative effect on the cells. This mixing system can also be used with other grafts or factors and presents a reliable method for enrichment of bone grafts with biologics. Bone grafting for defect filling has become part of the surgical routine and optimization of this technique, like mixing with antibiotics or stimulating factors, will be a relevant issue in the future, allowing an individualized treatment.
It has been proposed that localized and controlled delivery of alendronate to osteoporotic femur via tissue engineering scaffold may be a valuable adjunctive treatment for fracture prevention. The objectives of this work were to develop an Alendronate loaded controlled release nanocomposite plasticized gelatin as glue that would form a suitable matrix supporting osteoblast proliferation and differentiation. Alendronate release was successfully controlled, with complete suppression of the burst phase of release by intercalation of alendronate anions in calcium/aluminum layered double hydroxide (LDH) clay nanoparticles and dispersed in the 10% gelatine matrix solution. XRD, TTIR and SEM were utilized to structural study of the scaffold/carrier. The Drug release was measured with HPLC and FTIR. Also the release process was modeled with graded buffer varied in pH. MTT assay, Alkaline Phosphatase measurement and Hematoxilene-Eosine cell staining were used to study the in-vitro behavior of human primary osteoblasts. Finally the results illustrated that LDH-based Scaffold can be an appropriate alternative for Alendronate carrier.
The collagen-liposomes conjugates were found to deliver higher levels of active agent over a sustained period of time, *in vivo*, compared to normal collagenous preparations. The aim of this study was microscopy characterization of a new composite made from collagen, liposomes and *Urtica dioica* derived polyphenols (Pf), in order to use it in the local treatment of rheumatic and inflammatory disorders. Positive charge liposome containing Pf were prepared by thin film hydration method using a suitable lipid mixture of phosphatidylcholine, dioleoylphosphatidylethanolamine, cholesterol and stearylamine in 4:2:3:1 molar ratio (8.5 mg/ml lipids). Multilamellar vesicles were formed spontaneously by gently mechanical shaking of the flask upon hydration of the dry thin lipidic film with phosphate buffer saline, pH 7.4 containing plant (*Urtica dioica*) derived polyphenols (3mg/ml). In order to fabricate the composite, a solution of collagen type I (5.3 mg/ml) was mixed with a solution of liposome containing Pf, in a ratio of 1:1 (v/v). The mixture was gelled at room temperature. Gels were frozen at -40°C and freeze-dried 24 h, for obtaining porous composites. Both, gel and porous composites were used for light and ultrastructural studies. In the presence of collagen type I, Pf-coated liposomes were entrapped within the fibril network. Moreover, collagen fibrils appear to be firmly attached to the liposome surface, suggesting the existence of interaction between collagen and liposome membrane. Microscopy studies have demonstrated that both, in gel and porous composites, collagen interacts with *Urtica dioica* derived Pf coated liposomes suggesting their use as intra-articular drug delivery system.
Optimization of Chitosan Nanoparticles for Anticancer Drug Delivery

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A great challenge in the biomedical and pharmaceutical fields is the development of systems that could encapsulate and deliver specific drugs in a localized and controlled way. In particular, drug-loaded chitosan nanoparticles may be easily decomposed in biological environment, promoting the diffusion of drugs in targeted way with beneficial therapeutic effects. In this work, the optimization of chitosan nanoparticles is proposed by loading two different drugs, i.e. Tetracycline and Doxorubicin - frequently used in different cancer therapies. Chitosan with a high degree of deacetylation (75-85%) was processed via electrospraying by properly setting polymer concentration and process parameters to obtain sub-micrometric round-like particles. Particle size and shape were investigated by image analysis on images from FESEM while the drugs release was investigated in different media at several pH values - from neutral to acid environmental conditions. Drug loaded chitosan particles show an average size ranging from 0.28 ± 0.05 μm to 0.64 ± 0.15 μm as a function of the imposed process parameters. Release curves characterized by the presence of an initial burst followed by a sustained release during the first 24 hours in PBS (pH 7.4) have been detected independently upon the particle izes. Moreover, the peculiar chitosan chemistry assures a faster release of the anticancer drug moving toward acid environment by a fine control of the pH conditions. All these preliminary data are really promising to design nano-shuttles for targeted drug delivery in different applications of cancer therapy.

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Injectable Hydrogel Developed Using Porcine Articular Cartilage

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Recently, considerable effort has been devoted to the development of several administration routes for protein drugs. An injectable hydrogel can be used to achieve greater therapeutic efficacy than conventional drug delivery such as oral or intravenous administration for protein drugs. In this work, we developed a delivery system capable of maintaining a sustained release of protein drugs at specific sites by using potentially biocompatible injectable extracellular matrix hydrogel. The porcine articular cartilage powder (PCP) was easily soluble in phosphate-buffered saline. The PCP suspension easily entrapped bovine serum albumin (BSA) as model protein. When the BSA-loaded PCP suspension was subcutaneously injected into rats, it formed an interconnecting three-dimensional PCP hydrogel. The release of BSA from the in vivo PCP hydrogel was sustained over extended experimental periods. In conclusion, this finding shows that the PCP hydrogel could serve as a minimally invasive therapeutics depot.
Development of Drug Release Systems of Composite Microspheres of Chitosan and Nanoclays

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Pharmaceutical nanoparticles are often made of organic polymers but inorganic systems are receiving much attention. Polymer/clay nanocomposites are a class of hybrid systems in which inorganic or organo-clay nanoparticles are dispersed in a polymer matrix. Clays are characterized by a layered structure and exhibit properties such as good water absorption, swelling, adsorbability and cation exchange. They also provide spontaneous submicron dispersions in aqueous media, resulting in low cost and biocompatible systems with large surface area. These characteristics combined with the enhancement of the mechanical and rheological properties makes these materials suitable to produce controlled release systems. It’s reported that the presence of clay mineral particles in the polymer matrix promotes the retarded drug diffusion and improves the dissolution behavior. This work aims at the development of a controlled release system based on chitosan hydrogel composite microspheres and nanoclay. The microspheres were prepared using different nanoclays (natural and modified montmorillonite) dispersed in a chitosan solution. Morphological (SEM), structural (XRD) and physicochemical (FTIR) properties were evaluated. Swelling studies and in vitro degradation were performed in PBS (pH 7.4) solution and the mass change (Qₘ) with respect to time was determined. Additionally a model drug, acetylsalicylic acid, was incorporated and in vitro drug release tests were carried out in PBS (pH 7.4). The amount of drug released was monitored by UV-vis spectrophotometry and the release kinetics was assessed. The most promising microspheres were selected to produce a controlled and sustained release system.


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Carbon nanotubes (CNTs) have been introduced recently as a novel carrier system for both small and large therapeutic molecules. Most of the research on CNTs has focused on their potential for delivery of anticancer agents. In our work will discuss the efficacy of Mitoxantrone (antineoplastic drug, MTX) absorbed on multiwalled-CNT (MWCNTs) on human breast cancer cell line, MDA. MTX was adsorbed on oxidized MWCNTs by interactions with CNT carboxylic group and drug amine groups. The adduct has been characterized through Raman Spectroscopy, Thermo Gravimetric Analysis (TGA), and TEM. Trypan blue dye exclusion assay was used to investigate the cytotoxic effect of MTX adsorbed on CNTs. The results showed that Mitoxantrone loading on carbon nanotubes produced a reduction in MDA cell viability slightly lower if compared to MTX solution. Cell viability in the presence of MTX loaded carbon nanotubes was dose- and time-dependent. These preliminary results showed that MWCNTs-MTX are as much effective as the free drug in killing tumor cell line; its physico-chemical and pharmacokinetics properties may support its use as an in-situ neo-adjuvant and/or adjuvant cytotoxic device. Actually, we are studying the combinatory effect of MWCNTs-MTX and some antineoplastic gallium salts, with the purpose of obtain a reduction of therapeutic MTX dose, maintaining drug's efficacy.
Oligonucleotide drugs including siRNAs and antisense oligonucleotides are promising therapeutics for treatment of several diseases. Their mechanism of action is via modulating the expression level of a target gene. Although there are some oligonucleotide drugs in the market, most of them could not surpass clinical trials and failed due to off-target effects, hepatotoxicity, short half-life and low stability in the plasma. Therefore, a delivery system is crucial for oligonucleotide therapy in order to increase the stability of the drug and prevent its rapid excretion. Peptide amphiphile molecules forming nanofibers or spherical micellar structures have been indicated to be successful agents not only in tissue engineering and regeneration but also for drug delivery. In this study, we synthesized and characterized different peptide amphiphile molecules that can form complexes with oligonucleotides via electrostatic interactions. These complexes were shown to be biocompatible with viability assays and our preliminary results with confocal microscopy and fluorescence assisted cell sorting analysis demonstrated that these complexes are taken up by the cells successfully. Peptide-oligonucleotide nanocomplexes can be used for increasing therapeutic efficiency of oligonucleotide drugs.
Enhanced Bone Differentiation by Simvastatin Loaded TiO$_2$ Scaffold

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The aim of this study was to develop a carrier system on TiO$_2$ scaffolds using simvastatin (SIM) in alginate to enhance bone formation. Ultraporous TiO$_2$ scaffolds were submerged into SIM-containing alginate solution followed by centrifugation to remove excess alginate solution. Subsequently, scaffolds were immersed into CaCl$_2$ solution to allow gelation. Scaffolds were finally rinsed with milliQ water and air-dried overnight. Microstructure of scaffolds was visualized by scanning electron microscopy and Periodic acid-Schiff staining. The SIM release was detected by UV-Vis spectroscopy. Primary human osteoblasts were seeded onto scaffolds and cultured for 21 days. Secretion of bone and vascularization markers was investigated by Luminex. An evenly distributed alginate layer covered the entire surface of TiO$_2$ scaffold struts. Sustained SIM release was observed for up to 17 days. SIM loaded scaffolds induced higher secretion of osteocalcin and vascular endothelial growth factor compared to alginate coated scaffolds after 21 days. Alginate coating can act as a carrier for SIM delivery, inducing osteoblast differentiation. The combination of the physical properties of TiO$_2$ scaffolds with the osteogenic effect of SIM may represent a new strategy for bone tissue regeneration in load-bearing applications.
Peptide based hydrogels have been investigated for numerous biomedical applications, including drug delivery, regenerative medicine and tissue engineering. Our research aims to understand network interactions involved in releasing small molecules from peptide hydrogels, and establish a model for their mass transport behavior. In this work, transport behavior of the peptide hydrogels were investigated experimentally and diffusion coefficients of the small molecules were estimated using semi-empirical methods. Depending on the peptide concentration of the hydrogel system, we showed that mesh size of the nanonetworks and any physical interactions between the small molecules and fibrillar network altered their diffusion coefficients. Development of an engineering model to translate the effects of physical interactions into predictable diffusion coefficients and mass transport behavior can aid researchers in building controllable scaffolds for biomedical applications.
Combining Self-assembly and Phage Display to Develop a Targeted Nanodelivery System for Cartilage Therapies

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The present work focuses on a specific challenge of great clinical importance: targeted therapy for osteoarthritis (OA). The identification of molecules expressed exclusively, or at elevated levels, by cartilage cells (chondrocytes) in OA conditions might provide a strategy for targeted OA therapy by enhancing drug specificity. Towards this goal, we report the identification of peptide ligands, that bind selectively and with affinity to OA chondrocytes, using phage display, a technology in which a library of phage particles expressing a wide diversity of peptides is screened to identify those that bind the desired target. A random 12-mer peptide library, displayed on the surface of a filamentous phage (M13), was screened by biopanning against the surface of OA chondrocytes to identify peptide ligands specific for these cells. Healthy and OA chondrocytes for the panning experiments were isolated from cartilage samples obtained in local hospitals under pre-established agreement and from patients after informed consent. Isolation and expansion of chondrocytes was performed according to published procedures and their phenotype was characterized by FACS (CD44, CD26, CD10 and CD95), RT-PCR (aggrecan, collagen I, II and X and Sox9), immunohistochemistry (collagen I, II and X), SDS-PAGE and western blot analyses. The identified peptide sequences are being integrated into nanocarrier systems formed by self-assembling approaches and the potential of these targeted delivery systems is currently being tested \textit{in vitro}. This approach, if successful, will yield important insights into the regenerative mechanisms of cartilage and could be applied for developing more efficient and less invasive therapies for treating OA.
Liposomes are widely used as drug delivery agents due to their biocompatibility, biodegradability and their resemblance to cell membrane. They can be functionalized with a wide range of bioactive molecules including antibodies, antigens, receptors, peptides, and aptamers. Their suitability for functionalization together with their extensive encapsulation capacity make them attractive tools for development of targeted drug delivery systems with enhanced \textit{in vivo} stability and circulation time. Peptide amphiphile (PA) molecules can be integrated into the liposomal membrane without any additional chemical functionalization steps. Besides their facile integration, the versatility of peptide sequences enables diverse biofunctionality to the liposomal carrier. In this work, we developed liposomes of negatively charged 1,2-dioleoyl-sn-glycero-3-[phospho-rac-(1-glycerol)] (DOPG) phospholipid that were functionalized with a cell penetrating arginine-rich peptide amphiphile molecule. We aimed to study a) changes in physical characteristics (size, surface potential and membrane polarity) of liposomal membrane due to peptide amphiphile incorporation and b) detailed encapsulation of hydrophilic (Rhodamine B) and hydrophobic (Nile Red) drug models. After optimization of the encapsulation efficiencies of the liposomes, doxorubicin HCl and paclitaxel were encapsulated as hydrophilic and hydrophobic drugs, respectively. This study investigates the effect of peptide amphiphile integration on physical and chemical properties of liposomes such as its size, morphology, surface charge and membrane integrity and discusses the \textit{in vitro} bioactivity of peptide amphiphile integrated liposomes on MCF-7 breast cancer cells in the presence of drug loaded liposomes in terms of cellular uptake and cytotoxicity.
Biodegradable microspheres have been extensively employed in pharmaceutical formulations for many routes of bioactive agent delivery. Polymers in medical area can be used as pure material, copolymers, composites, blends or interpenetrating network (IPN) structures. Semi-IPN structures contain at least two different types of polymers which do not form covalent bonds to each other but one crosslinks among the similar molecules, while the other entrap between the crosslinked network matrix. In this study, chitosan-polyvinylpyrrolidone (CH-PVP) microspheres and films were prepared as semi-IPNs in different compositions by water/oil emulsification method. Chitosan was crosslinked with glutaraldehyde and PVP molecules were entrapped in the network matrix of chitosan. It was aimed to control the release rate by controlling solubility of PVP. Therefore, microspheres with various CH/PVP ratios were prepared. Mechanical and stability studies carried out with the films prepared with the same compositions showed enough strength for applicability. A model chemotherapeutic drug, 5-fluorouracil (5-FU), was loaded into microspheres and its release kinetics was studied. In order to determine the cytotoxicity of the prepared systems, pure polymers, free drug (5-FU) and drug loaded microspheres (CH:PVP-1:1) were cultured with MCF-7 (human breast adenocarcinoma cell line) cells. Cytotoxicity results indicated higher cytotoxicity for the free drug than entrapped drug as expected, but the drug loaded microspheres demonstrated higher cytotoxicity than the unloaded microspheres, due to the sustained release of the drug from the microspheres.
Nano Modifications for Sensitivity and Activity: pH Sensitivity and PEG’lation for Cancer Therapy

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Systems used in medicine may need modifications in nano level without changing their bulk properties. These systems can be diagnostic (as biosensors) and therapy (as drug carriers) devices as well as implant materials (as stents). In any case, sensitive molecules for the desired purpose are needed to be attached to the system. In case of drug delivery, the aim is to maximize the therapeutic effect of the drug and minimize the adverse effects. In order to decrease the side effects and locate the release in the tumor area, targeting systems became to scene. For this purpose, nano size carriers are modified with sensitive molecules. In active targeting drug carrier is modified with ligands or antibodies which are able to recognize cancer cells, while in passive targeting, stimuli responsive delivery systems are developed. Adding pH sensitive functionality on the carrier locates the drug in the tumor area since pH of the most solid tumors is in acidic region as 4.7 - 5.5. Meanwhile, rapid development of tumor by abnormal and poorly controlled angiogenesis leads porous-wall vessels with pore sizes between 200 nm to 2 μm which lead to enhanced permeability and retention (EPR) effect. On the other hand, covalent attachment of polyethyleneglycol (PEG) to nano carrier mask the carrier from the host's immune system, increase its’ circulation time and enhance activity. This talk will give a summary of polymers and activation methods used in the preparation of sensitive and active macro and nano systems.
Gene-activated matrices (GAMs) have shown potential in localized gene delivery resulting in bone tissue regeneration. Chitosan (CS) is a natural cationic polymer which shows promise as a gene delivery vector as it is biocompatible, biodegradable and capable of intercellular delivery of nucleic acids. The objective of this study was to assess the potential of polymeric (Mw 160kDa) and oligomeric (Mw 6kDa) CS for the delivery of osteoinductive genes to mesenchymal stem cells (MSCs) in monolayer and on a 3D collagen-based scaffold, thereby developing a GAM for bone repair. Optimal conditions for formulating CS-DNA nanoparticles including pH, CS:DNA ratio, DNA load, complexation time and temperature were determined. Positively charged particles with a diameter of <200nm that can fully complex DNA were chosen for transfection experiments. Four formulations of polymeric CS and six of oligomeric CS carrying the Gaussia luciferase gene (pGLuc) were assessed in vitro in MSCs. Sustained transgene expression of 5X10^5 RLU was seen up to day 10 post-transfection with both types of chitosan while further prolonged gene expression to day 14 was observed in the oligomeric group. This study has led to the development of vectors that facilitate highly efficient and sustained transfection of MSCs for the first time. We are now assessing these CS vectors in 3D collagen-based GAMs and have the option of tailoring the expression of different osteoinductive growth factors, such as BMP-2 and VEGF, to create an integrated natural material-based GAM suitable for repair of critical sized bone defects in vivo.
Preclinical Safety Evaluation of Adipose-derived Stem Cells Engineered by Baculovirus for Bone Defect Repair in the Porcine Model

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Previous studies showed that mesenchymal stem cells derived from adipose tissues (ASCs) can be genetically engineered by baculovirus (BV) to express bone morphogenetic protein 2 (BMP2) and vascular endothelial growth factor (VEGF). Implantation of the transduced cells helps to repair bone defects on rabbits. However, the effect of BV transduction on cell characteristics and in vivo immune responses are not clear. In order to advance this technology into the clinical applications, this study aims to assess the safety of BV transduced human ASCs. Stemness, proliferation and immunosuppressive ability of BV transduced human ASCs were analyzed by flow cytometry and BrdU cell proliferation assay. Tumorigenicity and chromosome stability were detected by qRT-PCR and CGH array. A Critical-size defect at the femora of mini pig was established to evaluate the in vivo immune responses. The peripheral blood mononuclear cell (PBMC) was analyzed by flow cytometry and cytokines in serum were analyzed by ELISA. BV transduction did not impair the ASCs' viability, proliferation, immunophenotyping and immunesuppressive ability. Furthermore, no gene expression changes of either tumor suppressor gene or oncogene and the chromosome was stable. For in vivo immune responses, CD4+, CD8+ and macrophage percentage of PBMC demonstrated similar responses from 1 to 28 days post-transplantation (dpt) in mock and BV-engineered group. The Th1/Th2 cell cytokine decreased from 1 to 28 dpt while TNF-α increased significantly from 14 to 28 dpt in BV-engineered group. The Th1/Th2 cell cytokine decreased probably due to the secretion of BMP2 by transduced cells as BMP2 can inhibit B- and T-cell lymphopoiesis and lead to decrease of cytokine secretion. The TNF-α increased significantly from 14 to 28 dpt, suggesting that BMP2 accelerated bone healing and remodeling. This study demonstrated BV vector is a safe gene therapy carrier and validated the safety of BV-engineered ASCs for tissue engineering. The results are crucial for future applications of the technique in the clinical setting.
Transient in vivo BDNF/GDNF Gene Overexpression Promotes Motoneuron Survival and Functional Reinnervation in a Ventral Root Avulsion Model in the Rat

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Axonal injury implied close to cell bodies of motoneurons, such as ventral root avulsion induces the death of the vast majority of affected motoneurons. Brain derived neurotrophic factor (BDNF) and glial cell-line derived neurotrophic factor (GDNF) are potent survival factors for damaged motoneurons, but if produced in situ in a non-regulated manner they induce axonal sprouting without functional reinnervation, a consequence known as the "candy store effect". In a lumbar ventral root avulsion-reimplantation rat model, we applied a plasmid-based vector system to induce transient expression of BDNF and/or GDNF in the close vicinity of the damaged motoneurons. Rat adipose tissue-derived stem cells (rASCs) were transfected in vitro with these constructs and applied in vivo around the reimplanted ventral root, embedded in collagen gel. Evaluation of expression kinetics in vitro showed high expression levels at early timepoints after transfection and a desired decline after 2 weeks. Spatiotemporally limited neurotrophic factor therapy induced not only the survival of the injured motoneurons, but promoted the regeneration of their axons into the vacant ventral roots, too. Morphological reinnervation was accompanied by considerable functional improvement of hindlimb locomotor activity. BDNF, GDNF and a combination of both factors induced a similar extent of reinnervation (See Fig.1). These findings provide evidence that damaged motoneurons require spatiotemporally expression of BDNF and/or GDNF to support their survival and regeneration.

Fig.1. Transient expression of BDNF and/or GDNF induces reinnervation

![Graph showing reinnervation results](image-url)
Safe and Efficient Angiogenesis and Functional Improvement after Myocardial Infarction by Cell-based Expression of Controlled VEGF Levels

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VEGF can induce normal or aberrant angiogenesis depending exclusively on the amount secreted in the microenvironment around each cell. To aid the clinical translation of this concept, we developed a FACS-based technique to rapidly purify transduced progenitors that homogeneously express a specific VEGF level from a heterogeneous primary population. Here we aim at inducing safe and functional angiogenesis in the heart by cell-based expression of controlled VEGF levels. Human adipose stem cells (ASC) were transduced with retroviral vectors and FACS-purified to generate populations producing either a specific (SPEC) or heterogeneous (ALL) VEGF levels. 70 nude rats underwent myocardial infarction by coronary artery ligation and 2 weeks later were treated at the infarction border with either 10^7 control, SPEC or ALL cells or PBS. 4 weeks later the ejection fraction was significantly worsened in all groups (ALL VEGF -13.2%; control cells -6.4%; PBS -8.1%) compared to SPEC VEGF cells (+1.1%). Vessel density was increased by both VEGF-producing groups. However, ALL cells induced numerous aberrant angioma-like structures, while SPEC cells yielded only normal and mature microvascular networks. A positive remodeling effect was observed specifically in the SPEC group, with significantly reduced fibrosis in the infarcted area. Controlled VEGF delivery by FACS-purified transduced ASC is a promising strategy to achieve safe and therapeutic angiogenesis to treat chronic cardiac ischemia.
A Cell-based VEGF Delivery Device for Controlled Angiogenesis

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Uncontrolled expression of Vascular Endothelial Growth Factor (VEGF) can induce aberrant angiogenesis. We achieved sustained but controlled VEGF release in ischemic myocardium by FACS-based purification of transduced adipose tissue-derived mesenchymal stromal cells (ASC). However, efficacy is limited by poor cell survival after injection. Here we aim to generate an engineered ASC-based patch to promote both cell survival and angiogenesis in the surrounding tissue. Two populations of transduced human ASC were generated expressing either uncontrolled (ALL) or specific homogeneous (SPEC) VEGF levels and cultured for 5 days onto collagen scaffolds in a perfusion-based bioreactor. A 7-mm empty scaffold was stitched underneath the patch to simulate vessel ingrowth into an avascular tissue. After subcutaneous implantation in nude rats, ALL cells induced some aberrant vascular structures, but SPEC-ASC caused only normal capillary networks. Purified VEGF-expressing ASC induced a significantly greater vascular in-growth both inside the patch (intrinsic vascularization) and in the empty scaffold beneath (extrinsic vascularization) compared to naïve ASC, both at 1 and 2 weeks (1wk: 1.2- and 1.6-fold; 2wks: 14.7- and 4.7-fold, respectively). Cell survival was also higher in the SPEC-VEGF patches compared to the controls generated by naïve ASC. Induced vessels were not only morphologically normal, but also functionally perfused, as demonstrated by intravascular circulating lectin staining.
Adipose-derived stem cells (ASCs) hold promise as a cell source for bone and cartilage engineering, but ASCs are inferior in terms of potentials for osteogenesis and chondrogenesis. Therefore, ASCs engineered with baculovirus (a promising gene delivery vector) for growth factor expression failed to heal the calvarial defects in New Zealand White (NZW) rabbits. Here we engineered the ASCs for expressing either bone morphogenetic protein 2 (BMP2) or transforming growth factor-β3 (TGF-β3), and seeded the cells into either PLGA or gelatin sponge (which stimulates engineered cartilage formation). We unraveled that ASCs expressing BMP2 or TGF-β3 exhibited signs of osteogenesis and chondrogenesis in vitro. After implanting into the critical-size defects in NZW rabbits, the BMP2-expressing ASCs resulted in better repair than TGF-β3-expressing ASCs, regardless of scaffolds. Also, the cells in the gelatin sponges led to significantly improved healing than those in PLGA scaffolds. At 12 weeks post-implantation, the BMP2-expressing ASCs embedded in gelatin sponges healed ≈86% of the original defect area, ≈61% of the defect volume and the bone density within the defect reached ≈69% that of the skull. Very strikingly, the ASCs/gelatin constructs triggered cartilage formation along the bone healing, indicating that the calvarial bone healing proceeded through the endochondral ossification pathway, rather than the intramembranous pathway. In summary, the baculovirus-engineered, BMP2-expressing ASCs seeded in gelatin sponges resulted in effective calvarial bone healing and represent a novel approach for calvarial bone engineering.
Highly Efficient Non-Viral Gene-Activated Scaffolds Incorporating Angiogenic and Osteogenic Genes Enhance Bone Tissue Regeneration In Vivo

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Treatments combining nanotechnology with gene and stem cell-based therapies on biodegradable extracellular matrices are increasingly showing potential in bone tissue engineering. In this study, the ability of nano-hydroxyapatite (nHA) particles, developed in-house, to act as non-viral vectors for delivery of plasmid-DNA when combined with our collagen-nHA (coll-nHA) scaffolds specifically tailored for bone repair, yielding gene-activated matrices (GAMs), was determined. In addition, coll-nHA-dual gene scaffolds (dual GAMs) containing both an angiogenic gene, VEGF, and an osteogenic gene, BMP2, were assessed for bone healing in an in vivo rat calvarial defect model. FACS analysis performed on nHA-transfected rMSCs demonstrated a transfection efficiency of 12\% and no cytotoxic effects were observed. When cells were applied to the coll-nHA scaffolds under osteogenic conditions in vitro, the dual GAMs exhibited significantly greater osteogenic potential when analysed using microCT, calcium quantification and histology compared to single-gene GAMs and non-transfected cell controls. When the dual GAMs were assessed \textit{in vivo}, the nHA dual GAM outperformed all other groups demonstrating significantly superior bone repair as early as 4 weeks post-implantation as determined using microCT and histomorphometry. This research has demonstrated the potential of using novel coll-nHA scaffolds as GAMs for therapeutic gene therapy while also being capable of simultaneously delivering numerous genes. This study underlines the effect of specifically tailoring GAMs for bone regeneration applications and furthermore, this novel delivery system may be used for the regeneration of numerous other tissues besides bone.
Sustained and Efficient Non-Viral Gene Delivery from Liposomal Surface Coatings of Metal Stents to the Injured Vasculature

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Delivery of large biomolecules such as plasmid DNA encoding for a therapeutic gene from stents holds great promise for addressing the limitations of currently existing (small drug eluting) stenting platforms or catheter based delivery systems. To this end, a model in vitro system was established to examine whether cationic liposomes could be used for gene delivery to human artery cells. Three different formulations were compared (DOTMA/DOPE, DDAB/DOPE or DDAB/POPC/Chol) to examine the effects of different cationic and neutral lipids on the transfection efficiency of lipoplex-coatings of metal surfaces. Upon completion of the characterisation and optimisation of the materials for gene delivery in vitro, these coatings were examined on a range of stents and deployed in a rabbit iliac artery injury model in vivo. Maximal transfection efficiencies for all coatings were observed on day 28, followed by declining, but persisting gene expression 42 days after stent placement, thereby, presenting liposomal coatings for gene eluting stents as treatment options for clinical complications associated with stenting procedures.
Advanced Osteoplastic Material: Gene-activated Bone Graft

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There are a lot of different pathologies leading to formation of bone defects or bone tissue deficiency. Current medical technologies and osteoplastic materials allow to reach successful results of treatment in majority of cases (small-size defects), however replacement of large bone defects still remains one of the most actual problem in field of maxilla-facial surgery, traumatology and orthopedics. In this regards, development of more effective – advanced – osteoplastic materials is demanded. The goal of our research was estimation in vitro and in vivo the efficacy of gene-activated bone graft (GABG) previously made with collagen/hydroxyapatite scaffold and DNA plasmids with gene encoding VEGF165. We incubated GABG with culture of multipotent mesenchymal stromal cells (MMSC) to determine the effectiveness of transfection in vitro by measurement of VEGF concentration in cultural medium. Experiment in vivo was performed with rabbits (n=34). We did the bilateral critical-size (10 mm) defects of parietal bones to every animal. GABG were implanted into defects of right parietal bones (experimental group) while scaffold without nucleic acids were used for left side of cranium (control group). Results were evaluated by CT and different kind of histological analyzes in 15, 30, 45, 60, 90, 120 days. We have shown the increase of VEGF expression by MMSC in case of their incubation with GABG. Transfection of “recipient bed cells” by plasmids of GABG was shown in vivo and that resulted to more pronounced angiogenesis and reparative osteogenesis in all time points in case of experimental group in compared with control. Now we are carrying out a clinical trial for assessment of efficacy GABG for treatment of patients with defects of jaws.
Accelerated Vascularization and Improved Tissue Formation in Critical-Size Bone Grafts by VEGF-Expressing BMSC

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Generation of clinical-size bone grafts is hindered by the need for rapid vascularization, as necrosis ensues in the construct core at depths greater than about 1.5 mm. Here we tested the hypothesis that increased angiogenic stimulation from within osteogenic constructs by Vascular Endothelial Growth Factor (VEGF) can significantly accelerate early vascular in-growth. Bone marrow stromal cells from NZW rabbits were retrovirally transduced to stably express rabbit VEGF and seeded in critical-size HA scaffolds (3x2 cm), which were wrapped in a panniculus carnosus flap and implanted ectopically. In vivo imaging with contrast-enhanced angio-MRI showed significantly improved perfusion as deep as two thirds of the total construct size already 1 week after implantation, compared with mock-transduced or naïve cells (see panel A in Figure). After 8 weeks, micro-CT analysis showed 2-fold deeper formation of dense tissue in VEGF constructs. Histological analysis revealed greatly increased vascularization throughout the whole thickness of VEGF grafts (see panel B in Figure). Interestingly, extensive microvascular networks were found in the core, whereas large arteries were present in the outer regions, showing the formation of a physiologically structured vasculature, with conductance vessels feeding into large capillary networks for nutrient exchange. These results suggest that the combination of cell and gene therapy approaches is a promising strategy for efficient and rapid vascularization of critical-size osteogenic grafts.

A. Angio-MRI (1 week)  B. Vessel Density-Inner Core (8 weeks)

Control  VEGF  Control  VEGF

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Use of Hydroxyapatite Nanoparticles for microRNA Delivery in Bone Tissue Engineering

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microRNA (miR) can control tissue repair patterns, suggesting an appealing base for tissue engineering applications. This study aimed to develop a pro-osteogenic therapeutic by combining miRs involved in the osteogenic differentiation of human mesenchymal stem cells (hMSCs) with in-house developed nanohydroxyapatite (nHA) particles previously used for therapeutic plasmid DNA delivery. miR-133a inhibits osteogenesis by targeting the transcription factor Runx2 (2) and was investigated within this study. A nHA-miR (nanomiR) delivery profile using reporter miR-mimics and antagomiRs yielded an uptake efficiency range of 18 to 50% as determined by flow cytometry. Cell proliferation assays demonstrated no toxicity with miR treatment and qPCR results for the functionality tests demonstrated that both reporter nanomiR-mimics and antagomiRs function successfully by knocking-down their respective targets. These results served to select a nanomiR working dose for further investigation of osteogenesis promotion using antagomiR-133a, where augmented levels of Runx2 and calcium deposition were demonstrated by qPCR and a Calcium Liquicolor kit respectively. Taken together, these results indicate efficient delivery of functional miRs to hMSCs from nHA particles without toxic effects, and the ability to engineer hMSCs towards enhanced osteogenesis by incorporating antagomiR-133a in the delivery system. We propose that this novel delivery system may be used for the regeneration of numerous other tissues in addition to bone.
Chordin and Noggin Silencing in ASCs Improves Osteogenic differentiation Markers in Presence/Absence of BMP-2 in vitro

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Adipose derived stromal cells due to the ease of isolation and readily availability, has been widely applied in bone tissue engineering. BMP-2, plays a role in osteogenic differentiation of MSCs in vivo. Chordin and noggin as two BMP-2 antagonists are assumed to positively contribute to improve the osteogenic differentiation potential of ASCs.

ASCs were isolated from male and female donors. After seeding they were treated with 20nM siRNA against noggin and chordin. By adding osteogenic medium w/o 100ng/ml BMP-2, the cells were osteogenically differentiated up to 26 days. The gene expression of chordin, noggin, BMP-2 and col1A1 applying rt-PCR on day 4, were quantified. Furthermore, ALP and cell number in two early time points and Calcium as a sign of mineralization in two late time points were quantified. The gene expression assay shows a pronounced silencing of the chordin and noggin. Col1A1 as an osteogenic factor increased expression over control. The cell number and ALP results depict raise in chordin and noggin silenced groups compared to the control. Mineralization graphs also confirm the previous observations. Results revealed that silencing of chordin and noggin increases the osteogenic differentiation potential in ASCs. However, Chordin silencing raises the osteogenic markers more than noggin. Moreover, donor dependency is evident that the male donor shows better mineralization with BMP-2 treatment.
Elastin-like Recombinamers for Gene Delivery Containing Bioactive Peptides

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The creation of a gene delivery carrier suitable to release the therapeutic gene in the site of action is a challenge in the nanomedicine area. Elastin-like recombinamers (ELRs) as recombinant artificial polypeptides whose sequence or a main part of it, mimics the repeated motifs found in natural elastin has been shown as a biocompatible material and a potential source for different applications such as gene delivery. The proteinaceous nature of ELRs allows the addition of cell interaction motifs through genetic engineering techniques. In this study, ELRs were molecularly designed in which cell penetratin peptides with positive charge at neutral pH and fusogenic peptides were incorporated in their sequence with the objective to obtain good transfection efficiency in cells. The polymeric constructions were produced, purified and characterized. The physical studies of the polymer-pDNA complexes such as particle size, z-potential, stability, complexation capability were accomplished. Their biocompatibility was also tested in terms of blood aggregation and cytotoxicity as well as the transfection efficiency in C6 glioma rat cell line. The results showed good physical features and biocompatibility regarding to the cell transfection requirements. Concerning to the transfection studies, tested through fluorescence microscopy, polymer-DNA complexes showed internal localization in the cell. The distribution of these nanocomplexes, was found either in the cytoplasmic space or inside of the nucleus.
Non-Viral Gene Delivery Using a Novel Minicircle Scaffold Matrix Attachment Region System for the Treatment of Recessive Dystrophic Epidermolysis Bullosa

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Advantages of non-viral gene therapy include high production rates of vectors and a lower risk of immune responses in the host. Disadvantages, however, can be seen in low efficiency rates of transfection and transient gene expression level. This study aims to enhance non-viral delivery systems focusing on the skin disorder, Recessive Dystrophic Epidermolysis Bullosa. In this study minicircle (MC) plasmids - supercoiled DNA molecules that are devoid of their bacterial elements – were prepared by transforming E.coli with parental plasmids containing an L-arabinose inducible PhiC31-based intramolecular recombination system, which removes and degrades the bacterial backbone resulting in minicircle plasmid (4KB) after purification. Furthermore, through sub-cloning, a Scaffold Matrix Attachment Region (S/MAR) sequence was inserted into the parental plasmid downstream of the recombination site giving rise to an S/MAR minicircle construct. These constructs were used to deliver a GFP reporter gene to human keratinocytes from healthy patients by nucleofection. Results showed gene expression was increased in comparison to parental plasmids alone over a period of 4 weeks when minicircle or S/MAR constructs were used. The former construct (MC) may offer immunological advantages over the parental plasmids, the latter (S/MAR) may offer additional benefits such as a more sustained gene expression due to its ability to attract the transcriptional machinery of the cell and its capability to be maintained as an episome resulting in long term transgene expression. Results from studies comparing the long term expression of our constructs by FACS analysis indicate higher levels of expression and viability of cells for S/MAR minicircle constructs over a period of 4 weeks in comparison to that of parental plasmid.
Diblock copolymers of poly(4-vinylpyridine) (P4VP) and poly(oligoethylene glycol methyl ether methacrylate) (POEGMA) were synthesized for the first time using RAFT polymerization technique for using as a drug/gene delivery system. Physicochemical properties such as effect of ethylene glycol units in OEGMA, chain length of hydrophobic P4VP block, pH, concentration and temperature on solution behavior of copolymers were investigated. Copolymer chains associated to form micelles at pH values higher than 5. Size of micelles changed depending on the block ratio of P4VP and POEGMA. Below pH 5, individual polymers were observed owing to the repulsion between positively charged P4VP blocks. Hydrophobic pyrene molecules were entrapped by individual copolymer chains at acidic pH values by hydrophobic methacrylate chain of POEGMA block. LCST value of thermo-responsive copolymers changed depending on the length and charge of P4VP block. Complexes of thermoresponsive copolymers with antisense oligonucleotides were investigated in a wide range of component ratio and temperature. Size of copolymer-oligonucleotide complexes changed significantly with the change in the ratio of components above LCST, while minor variations in size of complexes were observed below LCST. Effect of physicochemical changes on gene uptake efficiency by these polymers was investigated in cultured cells and gene expression efficiency was evaluated.

**Keywords:** amphiphilic block copolymers, RAFT polymerization, pH-responsive, temperature responsive, gene delivery
BMP-2 Gene Therapy in Bioprinted Bone Constructs

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A well-known stimulus of bone formation in the field of regenerative medicine and tissue engineering is bone morphogenetic protein-2 (BMP-2). In previous work, a non-viral gene delivery system for BMP-2 was used as a means to apply physiological doses over a period of several weeks. The bioprinting technology produces scaffolds that are accurate and reproducible in size, shape and pore geometry. Porosity reduces diffusion distances and allows blood vessel ingrowth, which is highly desired for constructing clinically relevant sized bone implants. In this study, we aim to improve the efficiency of the gene therapy by printing 3D porous constructs.

3D constructs (10x10x7 mm) consisting of alginate supplemented with mesenchymal stromal cells and ceramic particles were printed either in a porous or a non-porous/solid fashion. The cDNA coding for BMP-2 was included in the constructs. Porous constructs could be bioprinted and remained intact for at least 14 days in culture. Cells were efficiently transfected by the cDNA, and differentiated towards the osteogenic lineage as shown by elevated BMP-2 and ALP production. Porous constructs performed significantly better in producing BMP-2 than solid constructs. After implantation for six weeks subcutaneously in nude mice, osteogenic differentiation of cells, or bone formation could not be confirmed, which calls for an optimization of biomaterials used.

In conclusion, we show for the first time a model in which 3D printing and non-viral gene therapy are combined.
Effects of the Surface Charge of Adult Stem Cell Membranes on Gene Transfection Efficiency

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Recently, a number of new techniques have been developed to deliver foreign DNA into adult stem cells. Gene delivery using nonviral vectors has received a great deal of attention because of ease in preparation, stability, and safety, although the systems tend to have low efficacy. Polyethyleneimine (PEI) is one of the most extensively studied nonviral gene carriers. In this work, we compared the efficiency of DNA transfection in rat bone marrow mesenchymal stem cells (rBMSCs) and rat muscle-derived stem cells (rMDSCs) using the cationic polymer polyethyleneimine (PEI) as a gene carrier. The DNA/PEI nanoparticles exhibited positive surface charges, which were proportional to the N/P charge ratio. rBMSCs membranes exhibited more highly negatively charged than rMDSCs membrane. Transfection efficiency was evaluated on the basis of electrostatic interaction between negatively charged stem cell membranes and positively charged DNA/PEI nanoparticles. In conclusion, our results indicate that rBMSCs were more effectively transfected with DNA/PEI nanoparticles than were rMDSCs, reflecting the higher negative charge of rBMSC membranes that facilitate the interaction with positively charged DNA/PEI nanoparticles.
Matrix-assisted Sonoporation (MAS): Non-viral in vitro and In vivo Gene Transfer in Biocompatible 3D Matrices for Tissue Engineering

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Tissue regenerative gene therapies rely on controlled transgene delivery \textit{in situ} and should be compatible with stem cells and biomaterial application. Sonoporation enables minimally invasive, spatially controlled non-viral gene delivery \textit{in vivo}. Since this method relies on proximity of therapeutic DNA, microbubble contrast agents and target cells for efficient delivery, it was the aim of the study to develop a novel approach using all components embedded within a matrix. A protocol for Matrix-assisted Sonoporation (MAS) was developed and gene transfer efficacy was monitored using luciferase in C2C12 cells \textit{in vitro}, followed by osteoinduction using a BMP2/7 co-expression plasmid. Subsequently, MAS was applied \textit{in vivo} in an ectopic nude mouse model for luciferase monitoring of gene delivery and BMP2/7 induced bone formation using rat adipose derived stem cells. \textit{In vitro} data indicated successful gene delivery within matrices and expression for up to one week. Alkaline phosphatase assays demonstrated osteoinduction by MAS-based delivery of BMP2/7. \textit{In vivo} application matrix formulations showed effective gene delivery within matrices and expression for 14 days. Activation of the matrices with ultrasound displayed increased gene expression when compared to matrix implantation-mediated passive gene delivery, demonstrating the feasibility of the ultrasound trigger. Evaluation of MAS-mediated ectopic bone formation is still pending. We conclude from these studies, that this approach enables spatially controlled, minimally invasive non-viral gene delivery to target cells in 3D within matrices and can be harnessed for tissue engineering applications in the future.

This work has been funded by the EUROSTARS project E!5650.
Gene therapy can be described as the introduction of a functional gene into cells with a defective gene in order to treat diseases of genetic origin. In the early ages of gene therapy survey, the gene therapy studies were highly depended to viral vector. The use of viral vectors, because of their insuppressible drawbacks such as technical difficulties in producing the vectors, the high cost of production and the production of neutralizing antibodies which decreases their effectiveness, was limited. These type limitations were eliminated by the emergence of non-viral gene therapy vectors with nano-based device and approaches. In literature so far carbon based, dendrimer, natural polymers, polyethyleneimine-derived nanoparticles, cationic lipid based nanoparticles and inorganic based nanoparticles were employed in gene therapy studies. Herein, we developed magnetite-gold core/shell systems to diagnose and treat tumor cell. In the early studies, in order to gain the electrostatic interaction of negatively charged plasmid DNA and nanoparticle surface, gold surface was coated 2-aminoethanthiol via gold-thiol linkage. To monitor the gene expression, plasmid DNA of genetically modified E.coli for green fluorescent protein (GFP) was evaluated through classical procedure and standard plasmid kit. Plasmid DNA and nanoparticles were conjugated in different mass ratios. Gel electrophoresis images confirmed the binding of plasmid DNA to nanoparticles. Afterwards, plasmid loaded nanoparticles were transfected to L929 mice fibroblast cells in three different ways; i) no magnetic force ii) unprocessed magnet iii) microstructured magnet. Fluorescence microscope images depicted that no transfection for the case of no magnetic force. Interestingly, microstructures on magnet remarkably increased the efficiency of transfection. Cell viability and fluorescence microscope images confirmed that depending on the nanoparticle-plasmid conjugate concentration cell necrosis was observed especially for microstructured magnet (46% necrosis for the highest nanoparticle concentration (16 µg/ml)). Plasmid loaded nanoparticles containing cells were incubated for 48 hours and presence of GFP confirmed the gene expression via fluorescence microscopy.

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Gold nanoparticles are effective tools to transfec cells for gene therapy. Here, gold particles are conjugated with transferrin in order to target estrogen receptor specific antisense oligonucleotides. Gold nanoparticles are activated by thiol groups which enabled the attachment of both a protein and an oligonucleotide. Gold particles in cells were visualised by microscopy. Cell proliferation was detected by MTT and estrogen receptor expression was measured by real-time PCR. Human breast cancer cells expressing estrogen receptor, MCF 7 have efficiently taken up these conjugates posibly though the uptake of transferrin receptors. The antisense oligonucleotides againts estrogen receptor were able to suppress cell proliferation as well as estrogen receptor expression. Further studies are being carried out using this delivery system against other overexpressed breast cancer specific genes and their messenger RNA.

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Intravenous Injection of Granulocyte Macrophage-Colony Stimulating Factor Significantly Enhanced Articular Cartilage Repair by Microfracture

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Microfracture technique (MFx) is the most well-known clinical treatment for articular cartilage defect. However, MFx has a limitation that repaired tissues are mostly unstructured and consist of fibrocartilages with poor mechanical property because MFx have a limitation in the amount of bone marrow mesenchymal stem cells (BMSCs). MFx is thought to drain BMSCs to cartilage defect area to have its therapeutic effect. Therefore, we hypothesized that therapeutic effect of MFx could be enhanced by stimulating proliferation of stem cells in the bone marrow using hematopoietic cytokines such as granulocyte macrophage-colony stimulating factor (GM-CSF). This study investigated the effect of intravenous (IV) injection of GM-CSF on stem cell mobilization and cartilage repair after microfracture in a rabbit model of articular cartilage defect. Forty New Zealand white rabbits were subjected to full-thickness chondral defect on their knees and divided into 4 groups: (1) untreated control, (2) treated with IV injection of GM-CSF, (3) treated with MFx, (4) treated with MFx after 20 minutes from IV injection of GM-CSF. The amount of BMSCs drained in the defect area was measured by colony forming unit-fibroblast (CFU-F) test and their surface marker profile, by flow cytometry. The repaired cartilage tissues were retrieved at 4, 8 and 12 weeks after treatment and examined by histological/immunohistochemical observation and biochemical assays for collagen type II and sulfated glycosaminoglycans (GAGs). MFx after IV injection of GM-CSF showed significantly higher amount of BMSCs than the MFx without GM-CSF treatment. No significant change was observed in the surface marker profile of BMSCs by GM-CSF injection. In histological observations and chemical assay, MFx after IV injection of GM-CSF showed slightly better cartilage repair than MFx alone at 4, 8 and 12 weeks. Moreover, IV injection of GM-CSF without MFx showed also better cartilage repair than the defect alone with no treatment. This study showed that IV-injection of GM-CSF could improve therapeutic effect of MFx on the articular defect probably by increasing the amount of BMSCs drained in the cartilage defect after MFx.
Addressing Swelling Caused by BMP-2 in Human Bone Regeneration

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Worldwide, patients continue to suffer from lack of bone. Gold standard treatment is the use of autologous bone graft obtained from the patient. This bone source has a limited quantity and the quality is dependent on the individual patient. Thus, bone repair by tissue engineering systems has attracted broad attention. Despite the continuing development of hormones and other bone-stimulating molecules, bone morphogenetic proteins (BMPs) remain the most potent inducers of bone formation. In particular, BMP-2 is widely recognized to be a powerful osteoinductive factor. Endogenous BMP-2 is also important for normal bone homeostasis and is upregulated immediately following bone trauma by recruitment, proliferation and differentiation of osteoprogenitor cells. In the clinical setting, BMP-2 absorbed into a bovine collagen type I sponge has proven to be effective in the treatment of degenerative disc disease and fracture non-union. BMP-2 efficacy in a clinical setting is remarkably low and milligram doses of the growth factor are needed to obtain therapeutic results. One parameter is the vehicle used for BMP-2 delivery. We have previously demonstrated preclinical data on bone formation using a hyaluronan-based hydrogel in combination with BMP-2 with promising outcome. In the present pilot study the hydrogel was evaluated in patients for the healing of alveolar clefts. We show that BMP-2 at concentration of 250 ug/ml in the hydrogel carrier is osteoinductive and the bone healing results are comparable to autologous bone graft from the iliac crest. Previous clinical studies for alveolar cleft repair using type I collagen sponge in combination with BMP-2 needed five to six times higher BMP-2 doses to obtain a similar effect. The hyaluronan-based hydrogel gives a prolonged release of BMP-2, as compared to type I collagen. Induced gingival swelling was a major drawback, which lasted for two weeks. Noticeably, bone healing occurred despite swelling, wound dehiscence and exposure of BMP-2-hydrogel compound in treated patients.

Recent evidence by our group and others suggests that BMP-2-induced bone formation is largely dependent on stability of BMP-2 and its release kinetics, with a controlled release enhancing the effect. Long-term BMP-2 delivery increases bone-healing rates compared with short-term delivery at an equal dose is the BMP-2 is kept intact. We aim to optimize the delivery of BMP-2 by the use of two approaches (i) release from an hydrogel that controls release and (ii) through the simple addition of a natural extracellular matrix (ECM) glycosaminoglycan (GAG) that complexes and protects BMP-2 towards premature degradation.
Synergistic Effect of Dual Release of Chemokine and Growth Factor on Tissue Regeneration

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For regeneration therapy based on the natural self-healing potential of patients, it is practically important to manipulate the inherent ability of cells for their proliferation and differentiation which fundamentally contributes to the self-healing potential. However, only when the cells with high potentials are transplanted without considering any their local environment, the cell-based tissue regeneration cannot be always expected. This is because in the body, generally cells survive and biologically function by interacting with their local environment. Tissue engineering is a biomaterial technology to artificially create the cell environment. For example, if a key bio-signal molecule is supplied to the right place at the right time period or concentration, the body system initiates to physiologically function, resulting in the natural induction of cell-based tissue regeneration. Biodegradable hydrogels have been designed for the controlled release of growth factors to succeed in the growth factor-induced regeneration therapy. However, in case that key cells are not present around the target site to be regenerated, it is practically necessary to allow the cell to recruit to the target site. The controlled release of stromal cell-derived growth factor (SDF)-1 could enhance the in vivo recruitment of stem cells. The cells recruited by the SDF-1 release were activated locally at the regeneration site by a growth factor release, resulting in enhanced cell-based tissue regeneration to a significantly great extent compared with that of only the growth factor release. This paper discusses about significance of dual release of chemokine and growth factor in biomaterial-based tissue regeneration therapy.
An important regulator of chondrogenesis is basic Fibroblastic Growth Factor (bFGF). bFGF stimulates both proliferation as well as ECM synthesis of articular chondrocytes. However, the described action of bFGF with respect to articular cartilage repair remains controversial. Current cartilage repair therapies mainly focus on (matrix-associated) autologous chondrocyte transplantation. We have developed a system, where autologous chondrocytes are cultivated in a 3D fashion, resulting in the formation of chondrocyte spheroids which produce their own hyaline ECM matrix. These spheroids, co.don chondrosphere®, are used to treat cartilage defects, without addition of xenogenous matrices. The possible regulatory role of bFGF in this tissue engineered cartilage was examined using articular chondrocytes obtained from individual patients. In individual patients, highly variable bFGF serum levels were measured, which however, did not show any correlation with the proliferation potential of chondrocytes in monolayer culture. During the course of chondrocyte monolayer cultivation the cells dedifferentiated, but bFGF expression levels remained constant. Although transfer of the chondrocytes into the 3D cell culture system did induce redifferentiation of the chondrocytes, bFGF levels remained similar as observed in the preceding monolayer cultures. Prolonged cultivation of the chondrocyte spheroids as well as fusion of the spheroids mimicking stages after transplantation of the cartilage cell transplant, did not change bFGF levels. These data suggest that bFGF levels, although highly variable, do not influence chondrocyte proliferation or anabolic potential in the studied tissue engineered cartilage.
TGF-β3-Releasing Pharmacologically Active Microcarriers And Human Cartilage Microparticles To Enhance Hyaline Cartilage Formation By MIAMI Cells

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Current treatments for articular cartilage defects face various problems, including fibrocartilage formation, graft failure, tissue hypertrophy, and harvest site morbidity. Our goal is to create an engineered product that can support marrow-isolated adult multilineage inducible (MIAMI) cell survival and chondrogenic differentiation, suitable for hyaline cartilage repair. We hypothesize that combination of growth factor-delivering biocompatible/biodegradable scaffolds and human cartilage microparticles will direct MIAMI cell chondrogenic differentiation and the formation of hyaline articular cartilage. MIAMI cells isolated from swine bone-marrow were combined with i) pharmacologically active microcarriers (PAMs) releasing TGF-β3 or ii) human cartilage microparticles (100-200µm in diameter), or iii) a combination thereof. The chondrocytic differentiation of MIAMI cells in contact with the scaffolds was assessed by RT-qPCR and histological analysis of the neo-cartilage pellets formed in vitro. Use of PAMs releasing TGF-β3 or cartilage microparticles enhanced cell survival and resulted in an increased expression of aggrecan and a decreased expression of collagen-X. The strongest effect on cell survival and hyaline-specific cartilage gene expression was obtained with the combination of PAMs releasing TGF-β3 and human cartilage microparticles.

PAMs releasing TGF-β3 combined with human cartilage microparticles are a strong determinant of the chondrogenic differentiation of MIAMI cells with hyaline phenotype. This novel strategy holds great promise for overcoming the challenges of hyaline cartilage regeneration in the repair of injured joints.
Production of the rhBMP-2 in E.coli and Evaluation of its Osteogenetic Activity In vitro and In vivo

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Bone morphogenetic protein-2 (BMP-2) is osteoinductive growth factor which is critical role in growth and regeneration of bone tissue. In clinical case, commercial collagen sponge on the basis on mammalian cell origin recombinant human BMP-2(rhBMP-2) is applied to spinal fusion procedures and acute, open fractures of the tibia. Because of low yield and high cost, it has limitations to widely use and rapid degradation is a critical shortcoming of collagen sponge scaffold. In this study, to overcome limitations of economical efficiency of mammalian cell origin BMP-2, the rhBMP-2 was clone to Escherichia coli expression system and cultured by fermentor at large scale and evaluated osteogenetic activity of in vitro and in vivo. The optimization of these conditions in an E.coli culture yielded 60g of wet cells per liter. After collecting of inclusion bodies, rhBMP-2 was refolded in 0.5M Guanidine- HCl by simple dilution method. The rhBMP-2 dimerized was purified by affinity chromatography. The purified rhBMP-2 dimer showed biological activity of differentiation into osteoblasts and induced alkaline phosphatase(ALP) activity in C2C12 cells. After 3 days of purified BMP-2 treatments(100,300,500 or 1000ng/ml), C2C12 cells showed changes in morphology like osteoblast and increases the degree of ALP. To investigated whether rhBMP-2 induce differentiation of MSCs into osteoblast, rhBMP-2 was administered in guinea pig subcutaneously. Because collagen gels are flowable, suggesting the possibility of an easily injectable, biocompatible drug delivery matrix, 1, 10 or 100ug of rhBMP-2 was incorporated with 2% of non fibrillar collagen gel and injected to guinea pig back. Animals were killed 1 week and 2 weeks after surgery and the tissues were processed for histological examination. Histology demonstrated dramatically dose-dependent increase of expression of osteocalcin and osteopontin which are markers of osteoblasts.
Evaluation of an Osteoinductive Calcium-phosphate Ceramic with or without rhBMP-2 in Critical-sized Bone Defects of Rats

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The aim of this study was to investigate the ability of an osteoinductive biphasic calcium phosphate ceramic (BCP) alone or in combination with rhBMP-2 to repair a 14 mm critical-sized calvarial bone defect in 120 male Wistar rats. Animals received a PTFE ring left empty (sham) or filled with BCP (BCP-0, Xpand Biotech, 150-500µm granules) consisting of β-tricalcium phosphate and hydroxyapatite in 80:20 ratio, BCP loaded with 2.5 (BCP-2.5) or 5.0 µg rhBMP-2 (BCP-5.0) per implant. Animals were sacrificed 3, 6, and 12 weeks post-implantation, respectively. Histomorphometric measurements were performed on Goldner trichrome-stained decalcified sections by software-assisted anaysis (Osteomeasure, Osteometrics, USA). New bone formation was expressed as area percentage of bone in the total available space (% BV/TV, mean±SD). Macroscopically, implants appeared firmly attached to the calvariae. In the sham group, new bone formation 3, 6, and 12 weeks post-implantation was 5.36±0.66, 4.66±1.10, and 6.80±1.90 respectively. In groups grafted with BCP newly formed bone extended from host bone bed to the center of the defect. At 3 weeks, % BV/TV was 14.73±0.65 in BCP-0, 17.41±1.34 in BCP-2.5, and 15.04±1.10 in BCP-5.0. At 6 weeks, a slight increase in new bone formation was observed – 23.92±1.60, 20.14±2.60 and 21.26±2.90 in BCP-0, BCP-2.5 and BCP-5.0, respectively. At 12 weeks % BV/TV was evaluated as 15.76±2.50, 20.41±0.90 and 20.35±1.70 for BCP-0, BCP-2.5, and BCP-5.0, respectively. No statistical differences among the groups were observed at any time point. These results demonstrate a comparable performance of an intrinsically osteoinductive ceramic alone or in presence of rhBMP-2 in regenerating critical-sized defects in rats.
Complications related to regeneration of bone remains a significant clinical problem. Due to unexpectedly low bioactivity of bone morphogenetic protein (BMP) its clinical application is largely restricted by the need of doses exceeding the physiological ones by several orders of magnitude. Thus delivery systems were developed to achieve continuous low level availability of osteogenic growth factors to stimulate bone formation in absence of adverse off-site effects (Lienemann, ADDR 2012). On the other hand, strategies to improve the mobilization of mesenchymal progenitor cell (MPC), which have both a great proliferation potential and the capacity to form bone might have a major impact on bone healing efficiency. To determine novel MPC recruitment promoting factors we have established an improved in vitro 3D migration model, where cells can be exposed to bound and soluble molecular cues. This platform was validated using known MPC recruiting factors such as SDF-1, PDGF, or BMP-2 in combination with human MPCs or prospectively isolated, murine osteogenic cells. Next, a panel of growth factors which are known to be involved in the healing of other tissues or cell types was evaluated. Best candidates were implanted in critical sized murine calvarial defects. The healing of defects was characterized by longitudinal evaluation of bone formation as well as by histological and immunohistochemical means. We believe that cell-instructive matrices containing novel MPC recruiting growth factors is a promising approach to improve BMP-2 induced bone healing. Such findings will likely inspire the design of next generation cell-instructive materials by relying on the modulation of naturally occurring processes during tissue development and regeneration.
Sustained and Highly Tunable Delivery of Engineered VEGF from Optimized Fibrin Matrices Ensures Normal, Stable and Functional Angiogenesis

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Vascular Endothelial Growth Factor (VEGF) is the master regulator of angiogenesis. However, the induction of safe and stable angiogenesis requires sustained VEGF delivery for at least 4 weeks at homogeneous microenvironmetal doses. Controlled release of a matrix-bound factor is a convenient approach for clinical translation of this biological concept. Here we aimed to determine the requirements to induce normal and stable angiogenesis by controlled release of VEGF from a state-of-the-art matrix-bound system, based on transglutaminase (TG) reaction to bind the modified recombinant factor into fibrin gels. Since both VEGF dose and duration are a function of degradation rate, we determined a gel composition with an optimized TG-aprotinin concentration to delay gel degradation, ensuring both sufficient in vivo persistence and an adequate VEGF release rate. After implantation of such gels in mouse skeletal muscle, VEGF concentrations greater than 25 μg/ml induced aberrant angioma-like structures, but a 500-fold range of VEGF concentrations between 5 and 0.01 μg/ml consistently yielded only normal and mature capillary networks (see Figure), which were stable and well perfused by 4 weeks. Finally, this optimized fibrin matrix with 2 μg/ml of TG-VEGF significantly improved both perfusion and wound healing rate in a rat model of ischemic skin wound. In conclusion, in vivo release of TG-VEGF from fibrin hydrogels can be precisely tuned under optimized conditions to efficiently induce dose-dependent, stable and functional angiogenesis.

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<th>TG-VEGF Concentration (µg/ml)</th>
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<td>a) Negative control</td>
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<td>d) 5</td>
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Endothelial cells (CD31) = red; Pericytes (NG2) = green; Smooth muscle cells (SMA) = cyan.
Stromal Cell-Derived Factor-1 Induction of Vascularization in Ectopic Bone

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For healing of large bone defects simultaneous stimulation of osteogenesis and vascularization is highly desired. Here, we investigate the use of stromal cell-derived factor-1α (SDF-1α) to stimulate vascularization by endothelial progenitor cells (EPCs) and we propose a replacing role for seeded cells by this chemokine in ectopic bone formation.

Goat EPCs were characterized for their endothelial phenotype. Matrigel plugs supplemented with ceramic granules and a single application or a combination of SDF-1α, EPCs or goat MSCs were implanted subcutaneously in nude mice. In order to determine the timing of bone-onset, fluorochromes were administered at week 3, 4 and 5. Implants were retrieved after 1 week (n=17) and 6 weeks (n=6) and analyzed for vessel network formation or bone formation and fluorochrome deposition, respectively. Colony forming EPCs were characterized by double positive labeling with acLDL/Isolectin B4 and by CD31 expression. SDF1α stimulated network formation by EPCs in an angiogenesis assay and induced tubule formation in vivo. Vascular networks were most prominent in MSC/SDF1α laden groups after 1 week in vivo. Implantation of MSCs with either EPCs or low concentrations of SDF1α resulted in ectopic bone formation after 6 weeks. In both groups, bone onset was before week 3 of implantation. This study demonstrates that SDF1α stimulates network formation in vitro and vascularization in vivo. Furthermore, SDF1α addition was as effective as EPCs in a prevascularization strategy based on MSC seeded groups. These results hold promise for the design of larger cm-scale bone replacement constructs in larger animal models.
Platelet Rich Plasma Exerts Antinociceptive Activity by a Peripheral Endocannabinoid Related Mechanism

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In the Regenerative Medicine, platelet byproducts containing factors physiologically involved in wound healing, have been successfully used in the form of Platelet Rich Plasma (PRP) for the topical therapy of various clinical conditions since it produces an improvement in tissue repair as well as analgesic effects. The pain suppression role has been investigated. Measurement of endocannabinoids has been performed in the PRP and in the keratinocyte cell line NCTC2544 treated with PRP by Gas Chromatography and Mass Spectrometric analysis. A mouse model of acute inflammatory pain was used for monitoring the analgesic effect of PRP and its lipidic extract. Analysis of endocannabinoids and related compounds in PRP revealed the presence of a significant amount of anandamide, 2 arachidonoylglycerol, palmitoylethanolamide and oleoylethanolamide. In the NCTC 2544 cell line, under inflammatory conditions, PRP induced a significant production of these compounds by the cells. Studies in a mouse model of acute inflammatory pain induced by formalin injection demonstrated a potent antinociceptive effect that was observed following intrapaw injection of: 1. total PRP; 2. lipids extracted from PRP; 3. an endocannabinoid enriched lipid fraction of PRP. Antagonists of endocannabinoid CB1 and CB2 receptors, injected in the paw, abrogated the antinociceptive effects suggesting for this preparation a peripheral mechanism of action. PRP and PRP lipid extract exert a potent antinociceptive activity linked, at least in part, to their endocannabinoids and related compounds content, and to their capability of elevating the levels of these lipid mediators in cells.
Degeneration of intervertebral discs (IVDs) is a condition associated with inflammation and low back pain. InGell Labs has developed InGell-Gamma polymer loaded with Interleukin-1 receptor antagonist (IL1-Ra) for sustained attenuation of IL1beta mediated degenerative changes. The objective of this study was to investigate the release profile and the activity of IL1-Ra upon release over 6 weeks. IL1-Ra has been incorporated in InGell-Gamma polymer. The amount released in phosphate buffer at 37°C during 6 weeks was determined by the bicinchoninic acid assay. The activity of released IL1-Ra was estimated according to its ability to prevent the proinflammatory response to IL1beta stimulation in human dermal adult fibroblasts. The mRNA expression levels of proinflammatory markers were measured by qPCR. InGell-Gamma polymer gradually degraded during a 6 week period, in which the IL1-Ra was released quantitatively in a diffusion-controlled manner. According to the activity assay we established, 100 ng/ml of IL1-Ra is the lowest dose that completely prevents the proinflammatory response to IL1beta stimulation in fibroblasts. This amount of released IL1-Ra showed full activity for 4 weeks and a partial activity for additional 2 weeks. In this study we have shown that IL1-Ra is gradually released from InGell-Gamma polymer in the period of 6 weeks and during that time effectively attenuates proinflammatory response in vitro.

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The Use of Highly Porous Collagen-Hyaluronic Acid Scaffolds as carriers of Therapeutic Biomolecules

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The requirement for an enhanced regenerative capacity of biomaterials designed for the repair of large articular cartilage defects has led to the development of biomaterials incorporated with recombinant growth factors. However, controlled release kinetics with retention of growth factor stability is one of the challenges with such a system. The aim of this study was to investigate the potential of using collagen-hyaluronic acid (CHyA) scaffolds as delivery systems for transforming growth factor-beta 3 (TGF-β3). By initially using bovine serum albumin (BSA) as a model protein, release could be achieved through direct incorporation of the protein into the CHyA scaffold suspension prior to lyophilisation. Approximately 60% of BSA was released from the scaffolds after 21 days. The stability of BSA was investigated using high performance liquid chromatography and was found to be maintained following 21 days incubation. Cross-linking treatment (1-ethyl-3-3-dimethyl aminopropyl carbodiimide) led to reduction in the release rate of the protein from the scaffolds with approximately 4-fold decrease (p<0.01) in the release of BSA after 21 days. TGF-β3 was subsequently incorporated into CHyA scaffolds using a similar method and it was evident that approximately 65% of the growth factor was release after 14 days. TGF-β3-loaded scaffolds were assessed for their chondro-inductivity using mesenchymal stem cells (MSCs). It was evident that such scaffolds supported significantly higher chondrogenic gene expression (SOX9, COL2 and ACAN) and sulphated glycosaminoglycan deposition in comparison to blank scaffolds after 14 days. This demonstrates the potential of using such scaffolds as novel carriers of therapeutic biomolecules with controllable release kinetics through the use of cross-linking treatment.
Improved Biomechanical Function of Cartilage Tissue Engineering Constructs Stimulated with BMP-4/-7 Heterodimer

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In vitro engineered cartilage still reveals poor mechanical properties and the development of constructs with an adequate biochemical composition withstanding high loads remains a challenge. Members of the bone morphogenetic protein (BMP) subfamily are known to enhance extracellular matrix (ECM) production by chondrocytes. Surprisingly, mechanical consequences of BMP treatment of tissue engineering (TE) constructs still remain elusive. Aim of this study was to assess, whether stimulation with BMP-4/-7 heterodimer can improve mechanical and biochemical properties of cartilage TE constructs. Porcine articular chondrocytes were cultured in a collagen matrix over 28 days with or without 100 ng/mL BMP-4/-7 heterodimer. Indentation testing using the Very Low Rubber Hardness (VLRH) method and confined compression analysis informed about biomechanical consequences. Cartilaginous ECM deposition was detected (immuno-) histologically and quantified biochemically. BMP-4/-7 stimulation significantly increased ECM deposition. Enhanced GAG content correlated with enlarged volume, enhanced stiffness (E-Modulus) and compressibility (Poisson’s Ratio) of constructs. Hardness values (VLRH) reached ~78 % of native cartilage. This study is the first to demonstrate improved biomechanical function of cartilage TE constructs after stimulation with a growth factor of the BMP family.
Bisphosphonate Linked Hyaluronan-Based Hydrogel Increases Both In vitro Retention of BMP-2 and In Vivo Resistance to Enzymatic Degradation

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There are several disadvantages with the current collagen based carriers used for delivery of bone morphogenetic proteins (BMP). One such disadvantage is the poor retention resulting in the need to use very large doses of BMP. Our aim was to evaluate whether addition of bisphosphonate (BP) into a hyaluronan hydrogel will alter retention of BMP as well as the degradation of the gel. Gels with and without linked BP carrying BMP-2 were prepared and added into medium that was refreshed at several time points during 2 weeks and analyzed using ELISA. HA-gel released almost 100% of the BMP-2, while the BP-gel released less then 10% of the BMP-2. The in vivo performance of the gels was examined in a femoral defect in rat. The defects were filled with 40 µL of BP-gel (left leg) and the same amount of gel without BP (right leg). The gels were either carrying no BMP-2, low dose (0.2 µg) or high dose (6 µg) of BMP-2. The legs were analyzed by peripheral computed tomography, histology, and micro computed tomography after 4 weeks. Two-way ANOVA analysis showed that BP and BMP-2 doses did not interact. In addition, BP alone had no effect on the bone mineral content (BMC) or bone area (BA), but adding BMP-2 caused a significant increase of both BMC and BA that was not seen when comparing the two doses. A substantial amount of BP-gel was still present after 4 weeks, in contrast to gels without BP, which were completely resorbed. In conclusion, BP-gel had a higher retention of BMP in vitro and was more resilient to enzymatic degradation in vivo, compared with gel without BP. Surprisingly, these differences in retention and gel degradation did not affect the bone formation. Thus, a later time point could be of interest to measure the osteogenic effect of the remaining BP-gel.
Platelet rich plasma (PRP) related derivatives are widely used in orthopedic surgery with the promise of stimulating the anabolic process. The presence of growth factors in PRP is well documented, but its exact mode of action is unclear. Our aim was to set up a human in vitro model and test the biological effects of various blood fractions in the pathomechanism of bone ischemia. Bone samples were obtained from the removed femoral head during total hip replacements for primary osteoarthritis. Oxygen-glucose deprivation (OGD) was applied for 7 hours to model ischemic damage and cell viability was assessed at 3 and 6 days post ischemia. For quantitation the Methyl-thiazol-tetrasolium assay was used. Four blood fractions were added to the media during and after OGD: saline, native PRP, heparinized PRP, or the albumin-rich plasma pressed from platelet-rich fibrin which we termed SAAP. The effect of OGD was not prevented by the addition of either blood derivative, however, both heparinized PRP and SAAP markedly improved cell proliferation after OGD, while native PRP was without any effect. This is the first study to show that bone undergoing ischemia can be effectively supported by the anabolic action of blood derivatives. Surprisingly, only the activated SAAP or heparin-inactivated PRP had strong proliferative actions. This ex-vivo human study offers a new insight into the biological action of PRP and may shed light on the reason behind the heterogenous reports on its effectivity.
A number of conditions such as immunodeficiency, diabetic ulcers, venous stasis result in delayed wound healing that leads to severe complications. Since FGF-2 is a potent mitogen and chemoattractant for endothelial cells as well as fibroblasts, it appears to be a strong candidate as a potentiating agent for wound healing. However, the therapeutic use of FGF-2 is limited by the need to maintain its prolonged local release at levels sufficient to stimulate angiogenesis. Thus, the aim of this study was to develop functionalized collagen scaffolds for human FGF-2 delivery. The polymer based on crosslinked modified heparin had been developed for the incorporation of FGF-2 into scaffolds. Functionalized scaffolds were prepared from bovine collagen type I solution containing developed polymer by freeze drying. The data obtained by SEM and CLSM analysis revealed that the average pore size of developed scaffolds varied from 100 to 200 μm. Scaffolds containing heparin-based polymer were able to incorporate recombinant human FGF-2. The developed compositions promoted angiogenesis in the CAM assay. We have found the scaffolds supported growth and proliferation of the CHO-K1 cells. To evaluate the local effects of developed scaffolds loaded with FGF-2 in in vivo experimental model they were implanted subcutaneously into the ICR mice. Histological analysis, performed 4 month after surgery, revealed the migration of fibroblasts and ingrow of vessels and nerve fibers into the scaffold.
Photo-cured Hyaluronic acid/heparin-based Hydrogels Containing Bone Morphogenetic Protein-2 as a Bone Tissue Regeneration

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In this study, photo-cured hydrogel was prepared by aminoethyl methacrylate (AEMA) grafted hyaluronic acid (HA)/heparin, and then different concentrations of BMP-2 was loaded in the hydrogel. The methacrylated HA/heparin were prepared by reacting HA and heparin with AEMA. Methacrylated HA and heparin were dissolved in DI water and photopolymerized for 5 min. The effects of crosslinking degree on the physical-chemical properties of HA/heparin-AEMA hydrogels were evaluated by ATR-FTIR analysis, 1H NMR, release profiles, viability and differentiation test in vitro and in vivo. The effects of the hydrogel on osteoblast-like (MC3T3-E1) cells proliferation and differentiation were evaluated by cell viability, release profile, and Alizarin red assay. BMP-2 loaded HA/heparin hydrogel was observed sustained release in vitro. Photo-cured HA-hydrogel was successful carrier for sustained release of BMP-2 in vitro. It was observation that different concentration of BMP-2 loaded hydrogel enhanced cell viability and osteogenic differentiation of MC3T3-E1 cells in vitro. In vivo study, H&E staining, bone formation area showed that BMP-2 loaded photo-cured HA/heparin hydrogel yielded the most extensive bone formation among the group. There results demonstrated that BMP-2 was important osteogenic differentiation factor, and increased BMP-2 concentration promoted more osteogenic differentiation of MC3T3-E1 cells. BMP-2 loaded HA-hydrogel would be valuable for bone tissue regeneration.

Keywords: Photo-cured hydrogel; Hyaluronic acid; Heparin; Bone morphogenetic protein-2; Release kinetics; Bone formation
Scaffold and Scaffold-free Strategies towards Tendon Repair

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Tendon and ligament injuries constitute an unmet clinical need with approximately 100,000 new cases annually in US alone. Tissue grafts are considered the gold standard in clinical practice. However, allografts and xenografts can lead to potential disease transmission, whilst the limited supply of autografts in severe injuries and degenerative conditions restricts their use. To this end, scaffold and scaffold-free therapies are under development to address the tissue grafts shortage. Herein, we describe biophysical, biochemical and biological methods to maintain tendon derived cell phenotype and/or differentiation of other cell types towards tenogenic lineage; development of tendon-equivalent facsimiles; and ultimately functional neotendon formation. Growth factor supplementation was assessed as means to either maintain tendon derived stem cell phenotype or differentiate them towards tenocytes. The influence of conditioning media was assessed as means to differentiate skin fibroblasts and stem cells towards tenogenic lineage. Biophysical and biochemical/biological features were assessed as means to maintain tendon derived cell phenotype and directional neotissue formation in rat patellar tendon model. Rich in tendon-specific extracellular matrix cell sheets were produced by appropriate modulation of the in vitro microenvironment. Structural, biophysical and biological analyses were subsequently carried out. Treatment with 10 and 100 ng/mL of IGF-1 preserved tendon stem cell multipotency for up to 28 days in culture and minimised changes in marker expression and extracellular matrix molecules production enhancing that way the clinical potential of these cells. Hierarchically assembled collagen scaffolds and anisotropically ordered polymeric substrates of rigidity similar to native tendons facilitate tenocyte phenotype maintenance in vitro, whilst in vivo studies are under way to assess the extent of functional tendon regeneration. Appropriate modulation of the in vitro microenvironment of tenocytes with macromolecules enhances tendon specific extracellular matrix deposition within 6 days in culture, facilitating that way the wide acceptance of cell-sheet technology for tendon repair and regeneration.

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Fibrin, a natural polymer involved in the blood coagulation process, is formed from fibrinogen by the protease thrombin and is then polymerized to form a mesh. During wound healing it serves as reservoir for proliferating cells and growth factors. The aim of this study was to investigate the release kinetics of growth factors from fibrin gels made of varying concentrations of fibrinogen and thrombin and to evaluate proliferation and differentiation of mesenchymal stromal cells (MSCs) immobilized within these gels. Fibrin gels were functionalized with transforming growth factor beta-1 (TGF-b1), vascular endothelial growth factor (VEGF), platelet-derived growth factor AB (PDGF-AB) or erythropoietin (EPO) or with a growth factor mixture made from platelet lysate. After the gels had polymerized cell culture medium with and without MSCs was added and the constructs were incubated for up to 14 days in vitro. Supernatants were collected after different time points and concentrations of the released growth factors were determined by ELISA. Cell proliferation was assessed by lactate dehydrogenase and DNA content whereas cell differentiation was investigated by means of quantitative gene expression analysis. For TGF-b1, VEGF and PDGF-AB our data revealed a sustained release up to 14 days depending on the fibrin gel composition. In contrast, nearly the whole amount of EPO was released within 4 days. MSCs cultivated in platelet lysate functionalized gels showed highest proliferation rates compared to the other gels. From gene expression analysis differentiation of MSCs into several lineages can be assumed.
Reducing Lysyl hydroxylase 2 Expression in a Pro-Fibrotic Environment by Targeted Repression Using Zinc Finger Proteins

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Lysyl hydroxylase 2 (LH2), encoded by plod2, initiates pyridinoline (pyr) cross-linking of collagen. These cross-links are increased in fibrotic collagen. To date, no successful therapeutics have been developed to reduce pyr cross-linking in fibrotic disease. Therefore we aim to reduce LH2 expression by targeting transcriptional repressors near expression related transcription factor (TF) binding sites, to withstand induction by TGFβ1. To identify LH2 expression related TFs and their binding to the plod2 promoter, we performed promoter-reporter assays, inhibition of TFs, and chromatin immunoprecipitation in primary human dermal fibroblasts (HDFs). Several zinc-finger proteins (ZFPs) were developed to specifically target the plod2 promoter. These ZFPs were fused to various effector domains known to repress gene activity by altering epigenetic marks that promote gene expression. Transcription factors Sp1 and Smad3 were identified as important regulators of TGFβ1 induced LH2 expression. Targeting of ZFPs fused to transient repressor SKD near the binding sites of these TFs reduced TGFβ1 induced LH2 expression by more than half in HDFs. Currently we are targeting DNA methyltransferases to plod2 to introduce targeted DNA methylation in order to permanently reduce expression. To conclude, our data shows that targeting transcription related sites with effector domains fused to ZFP is a promising new strategy to specifically reduce gene expression in a pro-fibrotic environment.
Evaluation of Dual BMPs-immobilized Polydioxanone Porous Beads for Bone Regeneration

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Recently, autologous transplantation of cortical and cancellous bones has been considered as a gold standard for the effective bone regeneration. However, donor site morbidity and limited availability of donor materials are still remained as significant challenges in clinical field. In this study, we prepared dual BMPs (BMP-2 and BMP-7)-immobilized porous polydioxanone (PDO) beads for effective osteogenesis of mesenchymal stem cells and bone regeneration. PDO/Pluronic F127 porous beads were fabricated by an isolated particle-melting/melt-molding particulate-leaching method developed by our laboratory. The BMPs were immobilized onto the pore surfaces of the PDO/F127 beads via heparin binding. Their morphology, growth factor release behaviors, cell culture in the growth factor-immobilized porous bead, and the animal study to investigate the effectiveness of the porous beads for bone regeneration were conducted. From the growth factor release experiment, it was observed that the growth factors immobilized onto PDO/F127 porous beads were continuously released up to 21 days, for both BMP-2 and BMP-7. From the in vitro osteogenesis using BMSC as a model cell, the growth factors-immobilized porous beads showed positive effect for the osteogenesis which may promote the regeneration of defect bone, and thus can effectively treat the bone.

From the results, we could conclude that the BMPs (BMP-2 and BMP-7)-loaded porous beads may be a good injectable system for the effective bone regeneration.

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Hair follicle morphogenesis is governed by many kinds of receptors. It is therefore reasonable to ask whether some factor receptors like NGF-receptor are also involved in the regulation. Focusing on low-affinity-receptor [p75NTR] of NGF, we show that keratinocytes express p75NTR in an interesting pattern during murine hair follicle morphogenesis. In the long stages of hair follicle development p75NTR-immunoreactivity are seen in ORS of the hair follicle and dermal papilla fibroblasts from neonatal HF to hair cycle phase. In this investigation we found that p75NTR protein is expressed in murine back skin and its expression in HFs fluctuates with the transitions from neonatal to postnatal 56 days. Here we also show that p75NTR+ cells can give rise to new hair follicles and maintain most cell lineages of the hair follicle in vivo. These observations suggest that p75NTR is an important growth modulator during morphogenesis and remodeling of neuroectodermal–mesenchymal interaction systems like the hair follicle.
The basic fibroblast growth factor (FGF-2) plays an important role in proliferation and differentiation processes of a wide range of cells. However, a major obstacle to the application of FGF-2 is related to its instability during storage and delivery. Thus, new materials that enable to stabilize FGF-2 and control its release are of growing interest. It was found previously that FGF-2 was responsible for different cellular responses. The low, moderate, and high concentration of FGF-2 in the lens epithelial culture induced proliferation, cell migration or fiber differentiation, respectively. The ability to control the content of bioactive FGF-2 is of high importance. In the current paper we present the covalently crosslinked albumin/heparin assembly design for immobilization of FGF-2. The multilayer assemblies of oppositely charged albumin and heparin were constructed onto the gold-coated glass via layer-by-layer method at pH 4. After deposition of required number of layers, the system was crosslinked by glutaraldehyde and transferred to neutral Ph. The various methods of heparin immobilization have been undertaken. The binding of heparin to albumin was accomplished via multi or end point attachment. The obtained albumin/heparin coatings were exposed to FGF-2 solution. The adsorption of albumin, heparin and FGF-2 was monitored in situ by FT-SPR. The topography of the albumin/heparin assemblies was observed using AFM. It was found that the amount of FGF-2 adsorbed onto the albumin/heparin assemblies was depended on the concentration of the FGF-2 in solution, time of incubation, number of albumin/heparin layers.
Use of Autologous Plasma Rich in Growth Factors in Skin Wound Treatment as Coadjuvant to Conservative Therapies in Rabbits: Experimental Study

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Autologous preparation of Plasma Rich in Growth Factors (PRGF), is a novel therapeutic strategy for the acceleration of wound healing in a wide range of tissues, as it continuously releases multiple growth factors, including IGF-1, PDGF-AB, HGF, EGF, TGF-B1 and VEGF. Blood platelets have a major role in initiation of cutaneous wound healing. Cutaneous ulcers constitute a serious and rising problem, and are frequently difficult to treat. They involve big health costs each year. There is a great need for development of innovative and economic therapeutic approaches for ulcers treatment. The aim of the study is to evaluate the effectiveness of PRGF and wet wound dressing association in wound treatment.

A prospective, randomized study was carried out with 20 female New Zealand rabbits. Animals were divided in two groups, according to their survival times (7 or 14 days). Four identical surgical wounds were made on the back of each of the rabbits. Each wound received one of the following treatments:
- PRGF+Wet Wound Dressing (WWD)
- Saline Solution (SS)+(WWD)
- PRGF+Dry Wound Dressing (DWD)
- Control: SS+DWD

Macroscopic examination (wound healing (%), infection presence, scar colour, scar width and scar retraction) was carried out at 7 and 14 days.

Statistically significant differences were found between groups and between times, showing greater wound healing, decreased infection incidences and similar macroscopic appearance to physiological healed wounds in those that received PRGF and WWD.

The association of PRGF and WWD accelerates wound healing. PRGF resulted to be a safe, simple and economic treatment.
Platelet Lysate Functionalized Hydrogels for Tissue Regeneration Applications

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Over the last years various drug delivery approaches have been developed. Hydrogels, three-dimensional hydrophilic, polymeric networks, are capable of immobilizing bioactive factors. Platelet lysate (PL) can be obtained from platelet rich plasma (PRP) and denotes a natural source of growth factors (GF). The aim of this study was to establish and optimize a PL-based GF-functionalized hydrogel system evaluating GF loading concentration, optimized PL-hydrogel ratios and to investigate the GF release kinetics and the cytotoxicity of these gels. PL was generated by freezing/thawing the PRP, by activating the platelets with thrombin or by lysing the platelets with 0.5% Triton X-100. GF concentrations within the PL were determined by ELISA. In a next step, two commercially available hydrogels, Tissucol (fibrin gel, Baxter) and Histoacryl (cyanoacrylate, Braun), were mixed with PL and incubated for up to 28 days. Supernatants were collected after different time points and concentrations of the released GFs were determined by ELISA. To test cytotoxicity PL-loaded gels were incubated with medium for 24 hours. In parallel, human mesenchymal stromal cells (MSCs) were seeded into 48-well plates and allowed to adhere for 24 hours. Then medium was replaced by gel-conditioned medium and MSCs were cultured for additional 24 hours. The number of live cells was determined by DNA content and activity of lactate dehydrogenase. PL with the highest GF content was obtained by lysing the platelets with 0.5% Triton X-100. From Tissucol GFs were released up to 14 days whereas Histoacryl released GFs over 28 days. Viability of cells incubated with supernatants of Tissucol and Histoacryl was 100% and 80%, respectively.
Inflammation is one of the first crucial stages during the regular wound healing process. However, it can also lead to severe tissue damage in chronic wounds. In these pathogenic wound environments the ratio of inflammatory and anti-inflammatory cytokines is highly biased to the pro-inflammatory side. M1 macrophages are one of the key players for pro-inflammatory stimulation, which is enhanced in chronic wounds. In contrast, M2 macrophages are able to propagate matrix deposition and cell proliferation, as typically seen in the late stages of wound healing. However, the transition of M1 macrophages to M2 macrophages is delayed or disturbed in chronic wounds. The presence of additional anti-inflammatory cytokines could balance the disproportion between pro- and anti-inflammatory cues within the chronic wound environment.

Within the presented study we set out to correct this disproportion using cytokine releasing starPEG-heparin hydrogels, which have been shown to reversibly bind and release various cytokines and growth factors\textsuperscript{1,2}. IL-4, IL-10 and IL-13 are loaded onto starPEG-heparin hydrogels and their time-dependent release is monitored over 7 days. The anti-inflammatory effect of the cytokine containing hydrogel is tested to influence bone marrow derived macrophages, which are challenged by bacterial lipopolysaccharide treatment. The cellular response is examined via their cytokine secretion profile, the presence of reactive nitrogen species and the analysis cell surface marker expression.

With the presented hydrogel carriers we aim to establish a robust hydrogel system that can be translated towards a clinical use for the treatment of chronic wounds and diseases of uncontrolled inflammation.
Estrogen Effect on the Sclerostin Induction by BMP-2 in Human Mesenchymal Stromal Cells

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Introduction: Estrogen therapy decreases circulating levels of Sclerostin, a protein product of SOST which increase in postmenopausal women. However, the mechanisms of estrogen on the expression of SOST remain unclear. This study was hypothesized that estrogen modulates SOST expression by interfering bone morphogenic protein (BMP) signaling on the basis that BMP is an inducer of SOST in osteoblasts.

Description of methods: We investigated the expression of SOST and other BMP-2 responsive genes in the treatment either with BMP-2 (200 ng/ml), estrogen (100 nM), or combination of both using female-originated human mesenchymal stromal cells (hMSCs) by real time RT-PCR or ELISA. Molecular mechanism was examined using the inhibitor of Wnt (ICI 182, 780: 100 nM) and Smad pathway (AMPK: 10µM).

Results: There was no direct effect of estrogen on SOST expression, but estrogen significantly down-regulated SOST expression which was induced by BMP-2. Treatment with Wnt signaling inhibitor did not affect SOST induction by BMP-2, but counteracted the suppressive effect of estrogen on SOST induction by BMP-2. On the contrary, Smad inhibitor completely blocked SOST induction by BMP-2. This tendency repeated in the expression of other BMP-2 responsive genes such as alkaline phosphatase, BMP-2, or insulin growth factor-1.

Conclusions: Current findings suggest that estrogen regulated SOST expression by cross-talk with BMP-2 signaling. Estrogen suppressed SOST induction by BMP-2 through Wnt signaling.
Grafting Eptifibatide/NGF onto PCL Surfaces promotes the Proliferation and Differentiation of Bone Marrow Stem Cells into Neurites for Nerve Tissue Engineering

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It has been reported that the in-vitro proliferations of PC12 cells are promoted by grafting RGD analogue, Tirofiban, and nerve growth factor, NGF, onto PCL scaffolds for nerve tissue engineering\textsuperscript{1}. For developing biomaterials for nerve tissue engineering, it is of interesting to investigate the influences of incorporating a clinical RGD analogue, Eptifibatide (E), and NGF onto PCL surfaces (E/NGF-PCL) on the proliferations and differentiations of Bone marrow stem cells (BMSC) to neurites in-vitro. To graft E and NGF onto PCL surfaces, a photochemistry technique was employed. The successfully grafting E and NGF onto PCL surface was examined by FT-IR spectra and ESCA analysis. The grafting efficiencies of E (49.6%, surface density of $8.07 \times 10^{-4} \mu g/cm^2$) and NGF (98%, surface density of $2.47 \times 10^{-6} \mu mole/cm^2$) onto PCL surfaces were examined HPLC and ELISA methods. The results showed that the early adhesion (12 h) of BMSC on the E/NGF-PCL surfaces were significantly higher than that on PCL surfaces, examined by MTS and live/dead stain. For five days of cultivation, the proliferation of BMSC on E/NGF-PCL surfaces was significantly promoted with partially differentiated to neurites compared with that on PCL ones, examined by MTS and live/dead stain. Interestingly, after nine days of cultivation with 50μg/ml of NGF in medium for BMSC differentiations, the amount of the cells to differentiate to neurites on E/NGF-PCL surfaces were significantly more than those on PCL ones (e.g., around 3%) by examining the stains of Nestin and β-III tubulin of neurites/BMSC. In conclusion, grafting E and NGF to PCL to produce E/NGF-PCL surfaces could significantly promote both proliferations and differentiations of BMSC to neurites, and could be a promising biomaterial for nerve tissue engineering.
Benefits of Chemotactic and Inflammatory Modulators in Bone Regeneration

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Chemotactic and proinflammatory factors are secreted by various cells following injury. Although, their role in triggering regeneration was assessed as crucial in various tissues, many aspects regarding the effects of these factors on bone healing and their potential in bone tissue engineering strategies remain unclear. We aimed to evaluate the influence of these signals on bone marrow stromal cells' cytokines expression profile. For this, we assessed the mRNA expression levels of wide range of cytokines' mRNA in bone marrow stromal cells 72 hours after stimulation of cells for 2 hours with interleukin 1 \( \beta \) (IL1\( \beta \); 10ng/ml) or Stromal Cell-Derived Factor 1 (SDF-1; 1ug/ml) supplemented to the culture medium (DMEM, 10% Fetal Bovine Serum and 1% antibiotics). The results showed that the stimulation with certain factor regulated the expression of cytokines involved in various processes during fracture healing, including callus formation, remodelling, angiogenesis and bone cells differentiation. Specifically, IL-1\( \beta \) treatment induced the up-regulation of genes involved in angiogenesis (IL-6, IL-8) and osteoclastogenis (TNSF11), whereas down-regulated chondrogenic factor gene (GDF5). Stimulation of cells with SDF-1 positively regulated mRNA expression of bone formation factors (IL10, BMP8B) together with simultaneous down-regulation of IL-1\( \beta \). The effects of stimulation with IL-1\( \beta \) and SDF-1 on gene expression were consistent in cells obtained from different donors which indicate the importance of these factors for bone regeneration processes. Moreover, these results suggest that integrating inflammatory modulation in bone tissue engineering would provide more powerful strategy to enhance bone regeneration processes.
The bony insertion of soft tissue is described as an enthesis. The enthesis tissue includes collagen fibres that extend from the tendon through two specialized zones of fibrocartilage to anchor into the underlying bone. Tendon injuries are a common problem in both occupational and athletic settings. It is well established that the most failures of tendon are occurred at the bone-to-tendon junction, and the injured tissues typically heal slowly and thus frequently re-rupture with tendon retraction occur.

We fabricated dual growth factor (PDGF-BB for tendon and BMP-2 for bone)-immobilized asymmetrically porous membrane as a polymeric matrix to enhance the bone-to-tendon regeneration.

The amounts of growth factors immobilized on the membrane were quantified using an ELISA kit. The in vivo animal study to evaluate the bone-to-tendon regeneration potential of the dual growth factor-immobilized porous membrane was investigated using a patellar tendon defect rat model.

The both growth factors immobilized on the porous membrane were released with a sustained manner up to 35 days. For the animal study, it was observed that the dual growth factor-immobilized porous membrane allows greater bone-to-tendon regeneration than the other groups (membrane only & each growth factor-immobilized membrane).

From the results, we could suggest that the dual growth factor- immobilized asymmetrically porous membrane may be a promising strategy for the bone-to-tendon regeneration.

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Dual Growth Factor-Loaded Hybrid Bulking Agent for the Treatment of Urinary Incontinence

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Stress urinary incontinence (SUI), which is defined as the involuntary leakage of urine upon physical activity, sneezing or coughing, is considered a common and embarrassing problem among females. Various injectable bulking agents have been used in the treatment of SUI. However, the injection volume decrements with time caused by migration and resorption in the body have been considered as a critical problem. We prepared dual growth factor-loaded injectable bulking agent (bFGF-immobilized microsphere/NGF-loaded hydrogel) as an injectable and bioactive bulking agent which can provide bulking effect and stimulate the defect tissues around urethra for the effective treatment of urinary incontinence. Its growth factor release behaviours and the effectiveness of the bulking agent (using an urinary incontinence rat model) were conducted. It was observed that the growth factors (bFGF and NGF) were continuously released up to 30 days. The bFGF-immobilized microsphere/NGF-loaded hydrogel had a significantly faster smooth muscle regeneration and higher cure rate for the urinary incontinence than the groups with single growth factor.

From the results, we conclude that the bFGF-immobilized microsphere/NGF-loaded hydrogel system can be a good candidate as an injectable bioactive bulking agent for the effective treatment of urinary incontinence.

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Comparison between Penile Injection of bFGF-Hydrogel and Adipose-Derived Stem Cells for Enhancing Erectile Function Recovery in a Rat Model of Cavernous Nerve Injury

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Intra-cavernous injection of adipose-derived stem cells (ADSCs) enhances recovery of erectile function in cavernous nerve injury rat model. However, recent studies have demonstrated that intracavernously injected stem cells rapidly escaped the penis and showed limited effects. Basic fibroblast growth factor (bFGF) has effect on protecting arterial endothelium in the corpus cavernosum. To compare the effect of penile injection of ADSCs and bFGF Hydrogel on improving erectile function in cavernous nerve injured rats. Ten-week-old male Sprague-Dawley rats were randomly divided into five groups: normal group, BCNI group (bilateral cavernous nerve crush injury group), Hydrogel group (BCNI group with Hydrogel injection), bFGF Hydrogel group (BCNI group with bFGF Hydrogel injection), and ADSC group (BCNI group with ADSCs intra-cavernous injection). Functional test, immunohistological analysis, and molecular examination were performed four weeks after the operation. We detected the ratio of intra-cavernous pressure (ICP)/mean arterial pressure (MAP), and performed immunohistologic examination of bFGF, phallolidin, and CD31 expression in the corpus cavernosum. Cyclic guanosine monophosphate (cGMP) concentrations of the corpus cavernosum were quantified by cGMP assay. Both bFGF and ADSC treatment significantly elevated the ratios of ICP/MAP compared with the BCNI group. Only bFGF treatment group significantly increased bFGF expression in the corpus cavernosum compared to the cavernous nerve injury group. Furthermore, the expression of pallolidin, CD31 and the concentration of cGMP level in the corpus cavernosum were meaningfully increased in both of the bFGF and ADSC groups compared to the BCNI group. Penile injection of bFGF Hydrogel and ADSCs had equal effect on improving erectile function, inhibiting fibrosis of the corpus cavernosum in a rat model of cavernous nerve injury.

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Effects of Recombinant Human Epidermal Growth Factor (Nepidermin) Treatment on Inflammation during Diabetic Wound Healing.

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Deficient cicatrization of cutaneous diabetic ulcers is related to an extended inflammatory phase. Local treatment with Nepidermin (rh-EGF) has previously shown an improvement of the wound closure process in an excisional diabetic rat model. Our aim was to investigate the effects of this treatment on the process of inflammation in a diabetic wound healing model. Animals (n=64) were divided in 4 groups: healthy rats (HR), streptozotocin-induced diabetic rats treated with 0,5 (DR-0,5), 2,0 (DR-2,0) µg/mL Nepidermin or with placebo (DR-Plcb). A cutaneous excisional defect (Ø=1,5cm) was made, and peri-/intralesional injections of each treatment were administered 3 days/week for 14 days. Animals were sacrificed at days 3, 7, 14 and 21. Cicatization tissue samples were processed for histological and immunohistochemical analyses, and quantification (qRT-PCR) of mRNA levels of IL-2, IL-10, IL-12 and TNF-α. DR-Plcb group had an increased presence of macrophages and higher levels of cytokines (IL-10, IL-12, TNF-α) when compared to HR, suggesting a sustained inflammatory status. DR-0,5 and DR-2,0 groups followed an irregular pattern, showing a high number and activity of inflammatory cells at day 14, but resolving similarly to HR at day 21. No significant differences in IL-2 expression between groups were observed. Nepidermin treatment in diabetic excisional defects seems to induce a modulation of the dendritic cell and macrophage activity during the process of diabetic wound healing in an experimental model.

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Controlled Co-delivery of Engineered PDGF-BB and VEGF164 from Optimized Fibrin Matrices Normalizes Aberrant Angiogenesis Induced by High VEGF Concentrations

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Vascular endothelial growth factor (VEGF) is the master regulator of angiogenesis. However, delivery of safe and effective VEGF doses is challenging, as it remains localized in the microenvironment around each producing cell and it can induce aberrant angioma-like tumors if expressed outside of a narrow dosage window. In a gene therapy approach, we recently found that co-expression of the maturation factor Platelet derived growth factor-BB (PDGF-BB) can prevent aberrant angiogenesis by uncontrolled VEGF levels. Here we tested the hypothesis that controlled release of VEGF with PDGF-BB proteins from a state-of-the-art matrix-bound system, based on transglutaminase (TG) reaction to bind the modified factor into fibrin hydrogels, can prevent aberrant angiogenesis by VEGF. Taking advantage of optimized conditions we recently developed, we found that: 1) co-delivery of TG-PDGF-BB completely normalized aberrant angiogenesis induced by a high dose of TG-VEGF alone, yielding only perfused capillary networks instead; 2) normalization was achieved with a wide range of PDGF:VEGF ratios from 1:3 to 1:20; 3) normalization is reproducible at different high concentrations of VEGF, all of which induced abundant angiomas when delivered alone. These data suggest that controlled co-delivery of TG-VEGF and TG-PDGF-BB proteins is a convenient, safe and clinically applicable approach to expand the range of VEGF doses within which normal angiogenesis can be reliably and efficiently induced.
Functionally Graded Platelet Lysates Scaffolds Induce in vivo New Bone Formation

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Platelet lysates (PLs) provide an enriched source of growth factors (GFs) known for its stimulatory role on stem cells, that can also be used as a three-dimensional (3D) hydrogel. However, most of current PLs-based hydrogels lack stability, exhibiting significant shrinking behavior. Previous studies1,2 reported the development of novel 3D PLs-based scaffolds obtained by a supercritical fluid based methodology that have shown the ability to induce in vitro osteogenic differentiation of human adipose derived stem cells (hASCs) in response to a gradient of ceramic particles. The present work reports the in vivo assessment of such constructs using two different defect models in rats.

The reinforced PLs scaffolds were prepared by mixing the PLs suspension with genipin and Bioglass® microparticles and placed inside the high-pressure vessel, where crosslinking and the formation of a 3D matrix were achieved in a single step process.2 The constructs were implanted in rats up to 12 weeks both subcutaneously and in orthotopic critical size defects created in the ulna. Characterization of the explants included histological, immunohistochemistry analysis and Micro-Computed Tomography (Micro-CT). Subcutaneous implantation of PLs scaffolds demonstrated the long-term stability of the constructs, their ability to induce vascularization and lack of inflammatory response. PLs-Bioglass scaffolds were able to induce the formation of significant amounts of new bone leading to an almost complete bone bridging of the ulna defect region up to 12 weeks post-implantation. These results clearly demonstrate the potential of the newly proposed methodology to develop stable, biofunctional and autologous PLs hydrogels for orthopedic applications.
Age-Dependent Differences in Skeletal Muscle Regeneration After Injury

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The delayed functional and morphological recovery in the older rats more closely mimics the delayed recovery found in injured patients. Young MPCs transplanted into injured muscle of adult rats were able to enhance functional recovery. This model will provide a more physiologically relevant test for future cell therapy for the treatment of skeletal muscle injuries. Compartment syndrome (CS) is a serious complication arising from extremity injuries and resultant swelling within the muscle tissue. We previously developed and characterized a model of CS in young rats. The objective of this research was to characterize skeletal muscle regeneration after CS in older rats, which have a delay in functional recovery that more closely mimics the pathologies of CS seen in the clinic. Further, we examined the ability of muscle progenitor cells (MPCs) derived from adult and young rats to differentiate into mature functional myofibers and facilitate functional recovery after injury. Neonatal blood pressure cuffs were placed on the hind limbs 10-12 month old Lewis rats. A pressure of 120-140 mm Hg was held for 3 hours to induce CS. Maximum isometric force, as determined via neural stimulation, was used as the measure of muscle function. The tibialis anterior (TA) muscles were collected at days 14 and 28 after injury and processed for histological analyses. Our data demonstrated a delayed course of muscle regeneration in the older animals. Adult animals injected with young, but not adult, MPCs, showed significant functional improvement after injury. Animals injected with adult MPCs had greater fibrotic lesions and decreased MPC survival and integration.
The Effect of Immunocompromising Therapy on Musculoskeletal In vivo Tissue Engineering

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Muskuloskeletal tissue engineering is limited because of poor cell survival of transplanted myoblasts. Furthermore, granulocytes intake to bioartificial tissue (BAT) seems to be another barrier for impaired cell survival. Therefore in this study we were focused on the effect of immunocompromising therapy on transplanted myoblasts within a vascularized BAT in vivo. Myoblasts were harvested from 5 days old male Wistar rats and transfected with the sequence of Luciferase for in vivo monitoring with bioluminescence. Transfected myoblasts (4x10⁶) were seeded inside an in vivo bioreactor chamber within the abdominal wall of 4 weeks old male Wistar rats using the epigastric artery as central core vessel. Immunocompromising therapy was induced with daily administration of either cyclosporine or prednisolone and compared with animals without immunocompromising therapy. Bioluminescence monitoring of the Luciferase-transfected and in vivo transplanted myoblasts showed an increase of the signals from day 0 to day 7 in both groups with immunocompromised Wistar rats (cyclosporine: 26,51 10⁷ photons/sec on day 0 and 97,53x10⁷ photons/sec on day 7; prednisolone: 7,05 10⁶ photons/sec and 32,4 x10⁷ photons/sec). However, the signals in the control group decreased (1,47x10⁸ photons/sec and 9,69x10⁶ photons/sec) demonstrating a significant beneficial effect on day 7 with both, cyclosporine and prednisolone therapy. Cyclosporine and prednisolone have a beneficial effect on the cell survival of transplanted myoblasts in vivo and might therefore lifting musculoskeletal tissue engineering one step closer to clinical practice.
In Vivo Evaluation of the Effect of Human Mesenchymal Stem Cells (HMSCs) and Conditioned Media Associated with Different Vehicles in a Novel Skeletal Muscle Model

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Incomplete regeneration after traumatic muscle injury with residual functional deficiencies is a common problem in orthopedics and traumatology. Previously we developed a novel experimental muscle injury of the tibialis anterior muscle of the rat by standardizing a 5 mm-diameter myectomy lesion. Using this model we tested the application of HMSCs isolated from the Wharton’s jelly associated to different biocompatible vehicles to induce muscle regeneration. Sasco Sprague male rats with 250-300g were used. We tested different treatment combinations (HMSCs and conditioned media) and different vehicles (FloSeal®, a hyaluronic acid based hydrogel and fibrin glue). After 15 days, animals were sacrificed and the anterior tibial muscles were collected and fixed in formaldehyde. The International Standard (ISO 10993-6) for biological evaluation of medical devices was employed for assessment of the local effects after implantation of the different biomaterials used as vehicles in this study. The control and fibrin glue (with and without HMSCs) groups were also repeated in a different time-point (35 days) for evaluation of the muscles after complete regeneration.

In this study fibrin was validated as an appropriate vehicle for cells and other biological products, by obtaining scores below moderate inflammatory reaction in all the tested groups (both at 15 and 35 days post-surgery). In the groups treated with the conditioned media instead of MSCs a blunted inflammatory response seemed to occur. This finding may be justified by the fact that the conditioned media possesses a variety of growth factors and other molecules that result from the HMSCs in vitro metabolism during cell culture.
A New Preparation Method of Anisotropic Silk Fibroin Nerve Conduits and its Evaluation in vitro and in a Rat Sciatic Nerve Defect Model

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In the last decade silk fibroin (SF) has been emergently used in peripheral nerve tissue engineering (TE). Current approaches to form nerve guidance conduits (NGCs) are based on SF in aqueous solutions or organic solvents. Here, we describe a novel procedure that initially uses a braided tubular structure of raw Bombyx mori silk, which is further processed with the ternary solvent CaCl2/H2O/Ethanol, formic acid and methanol to improve its mechanical/topographical characteristics, leading to an anisotropic conduit favoring its use as an NGC. Mechanically an NGC has to withstand external forces from the surrounding tissue to guarantee room for the growing nerve stumps. Therefore, certain elasticity and kink characteristics are required. We could prove that this modification drastically improves the elastic properties of our tubular scaffold. In vitro, cell culture experiments were employed to exclude cytotoxicity (including Schwann-like cells) and the impermeability of the SF-NGC wall for possible invading cells. In vivo, the SF-NGC was tested in a rat sciatic nerve injury model with 1, 3 and 12 weeks observation time. Animals after 1 and 3 weeks served to prove the in vivo biocompatibility of the SF-NGC. After 12 weeks bridging nerve defect using the SF-NGC presented here, we could demonstrate a recovery of locomotor function comparable to autologous grafting investigated via gait analysis (Catwalk). The SF-NGC presented here shows promising potential for the treatment of peripheral nerve injuries. The modification of braided structures to adapt its characteristics may support the translation of SF-based scaffolds into the clinical setting.

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Electrophysiological, Immunohistochemical, and Functional Assessment of Regenerated Peripheral Nerve Using Nerve Guidance Conduits

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Autograft has long been regarded as the gold standard in peripheral nerve regeneration. However, due to limited availability of motor nerves and difficulties in using sensory nerves, nerve guidance conduits (NGCs) are becoming a more appealing alternative. This study examined the potential of NGCs to surpass autografts in terms of functional recovery. 75 rats (n=8) with gaps in the sciatic nerve of 10 mm or 15 mm were treated with hollow or filled NGCs or autograft. Recovery was assessed using morphometrical and immunohistochemical (IHC) analysis at 2 and 12 weeks respectively to evaluate regeneration of tissue within the lumen, reinnervated muscle mass to examine recovery from atrophy, electrophysiological measurements to assess restoration of compound muscle action potentials across the regenerated nerve, and force measurement analysis was used to compare levels of force recovery. IHC showed growth of NF160 labeled axons into the conduit and migration of S-100 schwann cells from both the proximal and distal stumps. Analysis of force measurement showed increased restoration of tetanic and twitch specific muscle forces in the filled conduit group across a gap length of 10 mm versus autograft (p<0.05). The EMG data also shows no difference in the electrophysiology of the treatment groups versus the autograft. This data shows that NGCs are not only becoming comparable to autografts for sub-critical and critical gaps, but may be beginning to surpass them.

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3D Micro-Printing of Nerve Guides for Peripheral Nerve Repair

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Nerve Guidance Conduits (NGCs) presently have a limited regenerative capacity, mainly due to the absence of physical guidance cues and poor support for nerve cell growth. The aim of this work is to develop NGCs with improved bulk properties, physical design and surface chemistry. Here the use of 3D structuring via laser stereolithography and electrospinning is reported for the fabrication of perineurial and epineurial mimicking scaffolds. Caprolactone, trimethylene carbonate and polyethylene glycol pre-polymers were synthesised using a microwave-mediated technique. These polymers were functionalised with methacrylate groups and characterised by GPC, MALDI-TOF-MS and NMR. The pre-polymers were UV cured into 2D sheets and 3D structures via stereolithography. Electrospinning of aligned PCL was undertaken with 5\textmu m parallel fibres inserted into NGCs. Characterisation revealed accurate and reliable production methods. \textit{In vitro} testing included culturing, cell viability testing and immuno-fluorescence labelling of neuronal cells, rat-derived primary Schwann cells and dorsal root ganglion, demonstrating cellular adhesion and neurite outgrowth on these materials. Early \textit{in vivo} implantation results of control microSL NGCs without lumen structures in to a mouse YFP axon common fibular model show regeneration equivalent to autograft. This work is continuing with the implantation of intraluminal-structured conduits. In summary, photocurable degradable polymers with 3D structures have considerable potential for the manufacture of a new generation of NGC, with improved physical and biochemical properties.
Fabrication of an Endoneurium using Engineered Neural Tissue within a Peripheral Nerve Repair Conduit

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Peripheral nerve injury as a result of trauma affects approximately 1 million people in Europe and America annually. The current clinical gold standard treatment for repairing long gaps is the nerve autograft, in which only ~50% of cases result in satisfactory functional recovery. Tissue engineered cellular bridging devices for peripheral nerve repair could provide an attractive alternative to autografts. Sheets of engineered neural tissue (EngNT), which is formed from columns of Schwann cells within a 3D aligned collagen matrix, can promote directed neurite outgrowth in vitro. These sheets of EngNT can be arranged to form the ‘endoneurium’ of a peripheral nerve repair device. Two different arrangements, rod-based and sheet-based designs, were tested within a clinically approved tube, NeuraWrap™, in a 5mm gap in the rat sciatic nerve (fig 1). Cross sections were stained to detect neurofilament after 4 weeks in vivo and revealed where the axons were growing in relation to the EngNT structures (this was divided into 3 zones for the analysis, fig 1). The axon density was significantly greater in zone 1 than in zone 3 in the devices (P<0.05, one-way ANOVA). The rod-based arrangement (A) gave a higher axon density in zone 1, 3350 ± 143 axons/mm² (mean ± SEM), compared to the sheet-based arrangement (B) (2920 ± 587 axons/mm²). The rod-based arrangement was more stable; there were no observed changes to its structure or orientation as a result of surgical handling or limb movement post-implantation. The designs are modular and can be adapted for the repair of bigger nerves by, for example, having multiple rod structures in the core of outer tubes or sheath wraps. Aligned cellular EngNT rods can form the basis of a functional conduit for peripheral nerve repair.

Figure 1. The different zones within a cross section of an EngNT device post-implantation.

(i) The different zones within the different device designs: EngNT rods (A), EngNT sheets (B), and the empty NeuraWrap tube (C). (ii) 15µm cross sections after 4 weeks in vivo in a 5mm gap in rat sciatic nerve, as observed on a light microscope for 3 different device designs. The red outlines the EngNT material and the blue outlines the device core.
Peripheral Nervous System: Neuro Tissue Engineering Using a Microstructured Collagen Matrix


Reconstruction of peripheral nerve lesions remains a major challenge in plastic surgery. Clinical strategies to treat extensive peripheral nerve injuries are currently limited to autologous nerve transplantations. As such, we here present alternatively a bioartificial collagen based scaffold with oriented micro channels providing biological, chemical and structural cues for regenerating axons. Schwann cell and dorsal root ganglia seeding were used to assess biocompatibility and axon-regeneration supporting properties of the materials In-vivo: Schwann cell seeded and cell-free scaffolds were implanted into 20 mm defects in the rat sciatic nerve, remaining 6 or 12 wks in-vivo. Long term cell-viability of Schwann cells was evident and cells displayed healthy morphology with spindle-shaped cell bodies and bipolar dendrites that orientated along the longitudinal channels of the matrix also at deeper layers of the scaffold. Regeneration experiments showed that sensory neurons originating from dorsal root ganglia oriented their axons along the longitudinal channels of the collagen matrix accompanied by supporting Schwann cells. In-vivo: After 6 or 12 wks, all nerve guides were well integrated into the host tissue. Intraoperative stimulation and electrophysiology of the regenerated nerves resulted in toe spreading and showed functional regeneration. Structural analyses presented myelinated and fascicular orientated axons reaching the distal part of the nerve graft already after 6 wks. Our collagen-based scaffold with oriented micro-channels was able to direct and support axonal regeneration in-vitro and in-vivo. As such, these current data provide sufficient support for first-in-human testing.
Tissue Engineering Peripheral Nerve Repair with Human Adipose Derived Stem Cells


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Adipose derived stem cells (ASC) offer tremendous potential for clinical applications due to their easy accessibility, multi-lineage differentiation properties and abundant expression of growth factors. In this study, we have investigated the neurotrophic and angiogenic properties of human ASC and assessed their effects in an experimental peripheral nerve injury model. Stem cells were enzymatically isolated from human abdominal adipose tissue and expanded in vitro. Stimulation of the cells with a mixture of growth factors resulted in increased secretion of BDNF, GDNF, VEGF-A and angiopoietin-1 proteins. Conditioned medium from stimulated cells increased neurite outgrowth of cultured dorsal root ganglia (DRG) neurons. Similarly, stimulated cells showed an enhanced ability to induce capillary-like tube formation in an in vitro angiogenesis assay. ASC were seeded into a fibrin conduit which was used to bridge a 10mm gap in the injured rat sciatic nerve. After 2 weeks, L4-L6 DRG and spinal cord segments were harvested and the effect of ASC transplantation on regeneration and apoptosis related gene expression determined. Immunohistochemistry using human cell specific antibodies showed the ASC survived transplantation and were found in close proximity to proliferating Schwann cells and regenerating axons. Both control and stimulated ASC enhanced the distance of axon regeneration and vascularisation of the conduit. Thus, ASC express neurotrophic and angiogenic factors, creating a more desirable microenvironment for regeneration in peripheral nerve conduits.
Auricular Cartilage Repair Using Engineered Neural Tissue as a Substitute for the Autograft in Peripheral Nerve Repair

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Engineered neural tissue (EngNT) has been developed comprising highly orientated Schwann cells that are functionally integrated within an anisotropic collagen matrix. This can provide a cellular guidance substrate for peripheral nerve repair when delivered within tubular conduits. In vivo tests in the rat sciatic nerve model showed that neurons regenerate within EngNT, with fibre diameters and myelin structures that are equivalent to those in an autograft (Fig 1). Conduits containing EngNT supported robust axonal growth across a 15mm gap and into the distal stump at 8 weeks, whereas empty conduits performed poorly in comparison. The Schwann cells within the EngNT provide trophic support to the regenerating neurons and are able to modify the extracellular environment through remodelling of collagen and deposition of matrix components. Because the formation of EngNT involves cells and native collagen fibrils aligning simultaneously via natural cell-matrix interactions, the resulting anisotropic material provides a simple tissue-like structure that can integrate easily with host tissue. The EngNT mimics the key structural and functional features of the nerve autograft and can be assembled using clinically relevant cells and materials. Autologous and allogeneic stem cells differentiated to a Schwann-like phenotype have been incorporated successfully into EngNT and the production process is suitable for scale-up and automation. Current research is focussed on developing the technology towards clinical application.

Figure 1: Neuronal regeneration within EngNT, rat sciatic nerve injury model, 15mm gap at 8 weeks
Neuroregeneration and Functional Improvement by the Transplantation of MSCs via Scaffold after Spinal Cord Injury

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This study aims to select the optimal method in transplanting mesenchymal stem cells (MSCs) for neuroregeneration after spinal cord injury (SCI). The injecting method is commonly used despite the possibility of inciting a secondary injury after the initial SCI. The scaffold can be a less invasive and more effective mediator of MSCs transplantation. The engraftment and differentiation of transplanted MSCs according to delivery methods were investigated. The functional recovery was also assessed. Sprague-Dawley rats were assigned to 4 groups; Group C (SCI only), Group IL (SCI + injection of MSCs), Group IP (SCI + MSCs via PLGA), Group IC (SCI + MSCs via chitosan). Allogeneic MSCs were directly injected via a 26G needle or applied onto the cord via scaffolds. After 6 weeks, immunohistochemistry was performed to identify the differentiated cell types (neuron, oligodendrocyte or astrocyte). Using western blot, neurotrophic growth factors (BDNF, NGF) were detected. Basso-Beattie-Bresnahan (BBB) scale was evaluated every week after injury. All data from Group IL, IP and IC were compared with each other and those from Group C. The number of completely engrafted MSCs was higher in Group IP and IC than that in Group IL. Differentiated cell types varied in respect to their delivery routes. Growth factors were expressed in larger amounts in Group IP and IC than in Group C. The improved BBB score was better in Group IP and IC than in Group C. The engraftment and differentiation of MSCs, and BBB score showed no significant differences between IP and IC groups. The scaffold is a safe and efficient mediator of transplanting MSCs into the spinal cord for inducing the neuroregeneration and functional recovery after SCI.
GAP-43 and Neurofilament Expression During Peripheral Nerve Regeneration as Quality Control in Tissue Engineering

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Nerve regeneration is characterized by axonal sprouting along the bands of Büngner, but the specific cellular and molecular processes that occur during peripheral nerve regeneration are not well understood. Growth-associated protein-43 (GAP-43) is a previously established marker of axonal growth during embryonic development. However, the relation of this protein during peripheral nerve regeneration using bioengineered nerve conduits and its relation with axonal regrowth remain unknown. The objective of this work is to evaluate the expression of GAP-43 and neurofilament proteins in a rat model of peripheral nerve regeneration. For this purpose, a 10-mm segment of left sciatic nerve was surgically excised under general anesthesia from each animal. The lesions of each animal were repaired using commercially available collagen conduits, and the nerve regeneration process was evaluated after 12 weeks. Finally, the expression of GAP-43 and neurofilament were analyzed by immunofluorescence. We observed that both proteins were expressed in the regenerated nerve fascicles, but they did not co-localize. GAP-43 was positive in thin axon sprouts of the regenerated fascicles with an axoplasmic pattern, and neurofilament was positive in thick regenerating axons with a cytoplasmic pattern. These results suggest that GAP-43 could be the responsible of guiding axonal sprouting of immature axons, and neurofilament will develop only after these axons become mature and well-established. Therefore GAP-43 could be used for quality control of nerve regeneration in tissue engineering. This work was supported by grant IPT-2011-0742-900000 from the Spanish Ministry of Science and Innovation (co-financed by FEDER).
Evaluation of Chitosan-Derivative Nerve Tubes for Peripheral Nerve Gap Regeneration

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Damages on peripheral nerves and consequent functional loss profoundly affect the social and daily life of a person. These problems are difficult to regenerate and sometimes impossible to treat. Within the scope of this work, in vivo effects of a chitosan and its poly-L-lysine derivative nerve tube on peripheral nerve damage have been investigated. The stimulating effects of the same derivative on in vitro nerve regeneration were previously shown. Rat sciatic nerve has been chosen as the model and an 8-mm gap on sciatic nerve has been bridged by the tubes made of chitosan and its derivative. Mechanical characterization was performed by uniaxial tension test in simulated body fluid. Functional recovery in animals throughout the 20-week follow-up period was evaluated by walking analysis and grip test. At the end of 20-week period, nerve regeneration was evaluated histologically. Results indicate that, although not equivalent to autologous nerve graft, application of chitosan/poly-L-lysine nerve tubes improve nerve regeneration in the defect site.
Chondroitin Sulfate Hydrogel for Nerve Tissue Regeneration

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Chondroitin sulfate (CS) has been employed as a biomedical polymer for tissue engineering of various tissues such as nerve, cartilage and bone because of its outstanding biological and structural properties such as biological activity, biocompatibility and biodegradability. In this study, after evaluation of biocompatibility of chondroitin sulfate hydrogel with PC12 cells, it was implanted in animal for its nerve tissue regeneration. The synthesized acylated and thiolated chondroitin sulfate powders were dissolved in two separate mediums, 10% (w/v) CS solution. After mixing two chondroitin sulfate solutions in separate medium, the CS hydrogel was spontaneously synthesized via Michael type addition reactions. While cell proliferation on and inside the hydrogel were observed with the assay of cell count kit-8 during cell culture, its viability was done by fluorescence microscopy using a Live/Dead assay kit. Cytotoxicity of the hydrogel was evaluated over the negative and positive controls of Teflon and Latex samples by the assays of MTT, BrdU and Neutral Red with PC12 cells. By adding different amount of the nerve growth factors in bolus and controlled manner, nerve cell behaviors such as proliferation and differentiation were in vitro observed both on the surface and inside the hydrogel. Control release of nerve growth factors was performed by incorporating them in the gel particles, which was in ranges of micron sizes. The hydrogels complex with nerve growth factors implanted in rats induced regeneration of nerve tissue over time as observed by histological staining. PC12 cells seeded on the surface and inside of CS-PEO hydrogels in vitro cultured for 7 days showed increases in cell adhesion and proliferation on the hydrogel. All the cells on the surface and inside the CS hydrogel were alive as confirmed by the results of the Live and Dead assay. The results of the assays of MTT, BrdU and Neutral Red demonstrated its excellent biocompatibilities when compared to those of Latex, a negative control. Their results were similar to those of Teflon, a positive one. Nerve cell differentiation was dependent upon delivery mode and amount of nerve growth factors. The hydrogel with growth factors induced significant amount of nerve tissues in rat. The hydrogel showed promising results for its applications in nerve tissue engineering.

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Keyword: Chondroitin Sulfate, Hydrogel, Biocompatibility, Controlled Release, Tissue Engineering
RGD Coated PCL Nerve Regeneration Conduits

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Peripheral nerve injury is a serious health problem for society today. Researchers have developed various strategies for recovery of nerve functions. Autologous nerve grafts are considered as “gold standard” for bridging long gaps, but this method has inherent disadvantages, such as limited supply, potential mismatch of tissue structure and size, donor site morbidity and need for multiple surgeries. Development of alternative treatments is necessary to bridge the gap between the proximal and the distal nerve stumps. To overcome the limitations of autografts, intensive researches are being conducted investigating artificial nerve conduits. The aim of this study is to produce biodegradable poly(ε-caprolactone) (PCL) electrospun fibrous nerve conduits coated with arginine-glycine-aspartic acid (RGD) cell recognition motif. For animal model studies biocompatible PCL nerve conduits with a 2 mm inner diameter and about 500 μm wall thicknesses were prepared by electrospinning. These scaffolds were further treated with Nap-FFGRGD (a molecule containing RGD tripeptide and hydrophobic naphthalene groups) thereby soaking in required solution. Morphology of the PCL nanofibrous scaffolds were characterized to evaluate the effect of solution system on nanofibers structure and to find suitable size of fibers, wall thickness of conduits, pore sizes for a successful peripheral nerve regeneration by scanning electron microscopy (SEM). Tensile testing of the films was used to assess the mechanical properties of PCL films and conduits. The thermal transitions of the conduits were obtained by a differential scanning calorimeter (DSC). The contact angle of the scaffolds was tested to assess hydrophilicity of PCL and PCL/RGD samples and get information about the effectiveness of RGD coating on PCL surfaces. Our results showed that by addition of RGD, water contact angle was decreased from 120° to 40° which could facilitate cells to attach and spread. The metabolic activity of L929 cells on PCL and PCL/RGD scaffolds was measured using WST-1 assay. Data shows that cell metabolic activity on PCL/RGD scaffolds at 48-h period was increased from 103,165 ± 17,991% to 114,460 ± 19,504%. Hoechst staining combined with propidium iodide (double staining solution) was used for necrosis quantification and morphological examination. According to double staining assay, the number of necrotic cells on PCL and PCL/RGD scaffolds are 12,026 ± 3,315, 7,206 ± 1,588 respectively.

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Regeneration of Peripheral Nerves by Transplanted Sphere of Human Mesenchymal Stem Cells Derived from Embryonic Stem Cells

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In cell therapy, the most important factor for therapeutic efficacy is the stable supply of cells with best engraftment efficiency. To meet this requirement, we have developed a culture strategy such as three-dimensional sphere of human embryonic stem cell-derived mesenchymal stem cells (hESC-MSCs) in serum-free medium. To investigate the in vivo therapeutic efficacy of hESC-MSC spheres in nerve injury model, we transected the sciatic nerve in athymic nude mice and created a 2-mm gap. Transplantation of hESC-MSC as sphere repaired the injured nerve significantly better than transplantation of hESC-MSC as suspended single cells in regard to 1) nerve conduction (sphere: 28.81±3.55 vs. single cells: 18.04±2.10, p<0.05) and 2) susceptibility of nerve stimulation at low voltage (sphere: 0.38±0.08 vs. single cells: 0.66±0.11, p<0.05) at 8 weeks. Recovery after sphere transplantation was near-complete when compared with the data of normal control (sphere 28.81±3.55 vs normal 32.62±2.85 in nerve conduction ; sphere 0.38±0.08 vs normal 0.36±0.67 in susceptibility of nerve stimulation, no significant difference, respectively). Recovery in function of the injured nerve was well corroborated by the histologic evidence of regenerated nerve. In the mechanistic analysis, the supernatant of sphere-forming hESC-MSC contains hepatocyte growth factor and insulin-like growth factor-binding protein-1 significantly more than the supernatant of the single cells of hESC-MSC has, which might be the key factors for the improved engraftment efficiency and greater regeneration of injured peripheral nerve.

Keywords: Sphere from hESC-MSCs, Peripheral nerve injury, Cell transplantation, Serum free- medium.
Nerve Guides with Different Architecture for Use in the Treatment of Spinal Cord Injury

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"Nerve guides" are used to bridge the neural gaps in the treatment of spinal cord injury, and therefore, the design of its inner architecture is very important in the treatment of the injured area. To study this, the effectiveness of the aligned and random media within the tubular nerve guides were compared. Although ultimately a tubular construct will be used, planar, multilayer structures were used to ease the monitoring and analysis. The bottom layer of the nerve guide, a PHBV foam, was prepared by freeze drying. Aligned and randomly oriented PHBV/collagen (2:1) fiber mats, to be placed on the base were made by electrospinning directly on the PHBV foam (Figure 1A). PHBV/collagen (2:1) foam to serve as a 3D disorganized medium was made by freeze drying. Crosslinking of the bilayer guides was made by dehydrothermal treatment at 150°C for 24 hours. Mercury porosimeter study showed that porosity of the PHBV foam was 85%, and the pore size was between 5 and 200 nm. Diameter of the fibers are measured by scanning electron microscope and found in between 200-900 nm (Figure 1B). In situ biodegradation test revealed that weight loss of uncrosslinked guides (no DHT) were less than the crosslinked ones (15% after 28 days), and therefore, crosslinking of the scaffolds was abandoned.

Figure 1: SEM micrographs of the nerve guides. A) Random PHBV/collagen (2:1) fibers (Fi) on PHBV foam (Fo) B) Measurements of the diameters of aligned PHBV/collagen (2:1) fibers.

It was concluded that the nerve guides prepared had proper porosity, pore size and degradation rate which would allow cell growth at the spinal cord injury site. Influence of the design of the guides will be determined after the seeding of the BMSC on the scaffolds.
Behaviour of Human Induced Pluripotent Stem Cell-derived Neural Progenitors on Laminin-coated Collagen Scaffolds

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Human induced pluripotent stem cells (hiPSCs) have led to an important revolution in stem cell research and regenerative medicine. Great potential to differentiate to variety of cell types and loss of ethical and immunonological problems are most advantages of these cells. Combining hiPSCs with biomaterial scaffolds provides a promising strategy for tissue engineering. Collagen and laminin are major components of the neural ECM that have a high impact in enhancing neural cell activity. Current study evaluated the influence of laminin, an important adhesion molecule for neural progenitors (NPs), on hiPSC-derived NPs behavior in a three dimensional collagen scaffold. The proliferation of NPs increased on scaffolds in a laminin concentration-dependent manner and 0.4% of laminin had more influence. The cultured hiPSCs-NPs on laminin-coated scaffolds highly expressed NP-specific markers after one weeks. Laminin coating promoted cell migration until 773±149 μm inside the collagen scaffolds with the 0.4% laminin concentration, which was about 16 times the cell migration in uncoated collagen scaffolds. Neural-specific gene expression experiments are ongoing. These results show that laminin in combination with a three-dimensional biodegradable collagen scaffold has a distinct effect on behavior of hiPSC-NPs, which has implications for cell therapies by tissue engineering.
Hierarchically Structured Poly-3-hydroxybutyrate Based Nerve Guidance Channels Enhance Oriented Axonal Outgrowth

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Peripheral nerve disruption resulting from injuries causes local insensitivity, paralysis and loss of autonomic control. Reconstruction of the severed nerve connections by exogenous means as a possible therapeutic approach still remains a major challenge. To stimulate peripheral nerve repair we developed a novel porous and degradable yet mechanically stable nerve guidance conduit (NGC) based on poly(3-hydroxybutyrate) (P3HB) which allows for oriented axonal regeneration. Porous tubular scaffolds were fabricated from P3HB blends with porogen by solvent-free thermoplastic processing followed by selective polymer leaching. The conduits maintained mechanical properties for over 16 weeks and at the same time allowed for diffusion of large molecules. Moreover, permeability and biocompatibility of the material were tested with in vitro studies of: 1- fibroblast survival and proliferation; 2- chicken dorsal root ganglia (DRG) and mouse sympathetic cervical ganglia (SCG) organ cultures as an in vitro model of axonal regeneration. Our results showed that NGCs successfully support cell survival, proliferation and neuronal outgrowth when closed for over 7 days. To induce the oriented neurite migration, fibrillar ECM-coated lumen fillers were implemented inside the conduit. This resulted in a remarkably increased ganglia attachment and directed axonal outgrowth. Altogether these results suggest that P3HB-based NGCs are promising scaffolds to be used in peripheral nerve gap repair strategies.
Precisely Patterned Model 3-D Bone Tissue Constructs

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Regeneration of bone tissue is a complex process that requires the involvement of multiple cell types. We hypothesize that introducing necessary cytokines and growth factors as well as cells capable of undergoing vasculogenesis and osteogenesis in well-defined spatial arrangements would enhance this process. Here, we report an inexpensive, high throughput technique to build in vitro 3D bone units using a novel photolithographic system with microscale precision. Aiming to mimic the complexity of bone, bone marrow stromal cells (BMSCs), human umbilical vein endothelial cells (HUVECs) and smooth muscle cells (SMCs) were encapsulated in a digitally specified 3D array. The precise control over the placement of cell encapsulated hydrogels would potentially enhance the diffusion of incorporated and secreted factors and the cross talk between angiogenic and osteogenic compartments, and also provide a platform for the real-time investigation of interactions between multiple cell populations. During the maturation and differentiation of vascular and osteogenic tissues, cell-cell interactions were investigated in the absence of external growth factors such as VEGF and BMP-2. The developed system allows for scale-up and the introduction of multiple cell types using a platform technology that is practical for individual laboratories to easily adapt using simple tools. We demonstrate the utility of this method by encapsulating cells in microgels aiming to form vascularized bone-tissue using building units in digitally specified and physiologically relevant geometries.
Functional Scaffolds for Tissue Engineering by De-novo Peptide Design

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The bottom-up design and assembly of scaffolds for tissue engineering will be useful in allowing tight control over the production of a complex extracellular matrix mimic. Here we present α-helical peptide hydrogels (Banwell et al. 2009), which we have decorated with an RGDS functional peptide to promote cellular attachment, proliferation, migration and differentiation. The three-peptide system produces gels at a physiological pH and temperature. Electron microscopy showed self-supporting gels consisting of long, thin fibres interconnecting to produce a porous network (Figure 1A and 1B). Circular dichroism confirmed that both the undecorated and RGDS-decorated systems were predominantly α-helical. In vitro cellular conditions maintained hydrogel stability over 14 days. Cell studies showed higher fibroblast and neural cell viability was promoted on the RGDS-decorated scaffolds (Figure 1C and 1D), with metabolic activity and DNA assays showing that the increased attachment lead to a higher proliferation rate on these gels. RGDS also promoted more and longer neural projections on the decorated gel. Cellular activity on undecorated gels formed directly beside RGDS-decorated gels showed that the neural stem cells migrated across the border towards the adhesion motifs. This study shows that RGDS-decorated hSAF gels are able to promote cellular adhesion, proliferation, differentiation and migration, and may be suitable as scaffolds for 2D and 3D cell culture.

Figure 1: SEM showed that Undecorated (A) and RGDS-decorated (B) gels were morphologically the same with DAPI and Calcein-AM staining showing cells remained viable on undecorated gels (C and D) with greater attachment on RGDS-decorated gels (E and F) on Day 14 of culture.
Self-Assembled Peptide Nanostructures for Controlled Cell-Materials Interactions

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We study concepts of making materials which mimic the structure and function of the extracellular matrix through programmed self-assembly of small molecules. The self-assembly mechanism forming the supramolecular aggregates involves non-covalent interactions such as hydrogen bonds, electrostatic and hydrophobic interactions. Diverse functional groups are incorporated into nanostructures, for example bioactive peptide sequences and metal chelating groups as well as hydrophobic motifs that include alkyl chains, steroid rings, and aromatic systems. The potential use of these nanostructures are studied for surface bioactivation of metal implants, extracellular matrix mimetic environments for desired cellular therapy and tissue regeneration studies.
RGDS-Poly(ethylene oxide)- Polydopamine Protein Repulsive Surfaces and Their Effect on Endothelial Cell Cultivation

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A simple versatile protein-repulsive substrate-independent biomimetic surface modification is presented that is based on the creation of a PEO brush on a polydopamine anchoring layer and brushe's capacity for selective follow-up modifications with various ligands using a copper catalyzed alkyne-azide cycloaddition reaction. The long-term stability of polydopamine-poly(ethylene oxide) surfaces can be improved through thermal annealing of the polydopamine layer [5] before poly(ethylene oxide) grafting. The desired surface concentration of biomimetic RGD peptide ligands can be controlled by adjusting the peptide concentration in the reaction mixture, then measuring the activity of 125I-radiolabeled peptides. that are immobilized on the substrates. The performance of the prepared substrates is tested in cell cultures with Bovine Artery Endothelium cell line.

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Laser-Structured 3D Scaffolds Improve Macrophage Adherence and Antigen-Specific Response

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The aim of the present study was to investigate the effect of scaffold’s surface topography on macrophage adherence, antigen presentation and specific antibody response development. 3D micro/nano structured Silicon surfaces comprising arrays of microcones were fabricated via direct laser structuring. Variation of the laser fluence leads to substrates exhibiting different topographical characteristics and wetabilities. Primary macrophages (Mφs) were isolated from BALB/c mice and cultured on such substrates. Mφs-coated substrates were seeded with antigen (Human Serum Albumin, HSA) and subsequently Th cells (isolated from BALB/c mice) were introduced to the culture. The adherence and recognition of Mφs and Th cells on the 3D surfaces was revealed by SEM and Confocal Microscopy. These microscopy experiments revealed that less rough 3D scaffolds surfaces were more favorable to macrophage adherence as compared to flat or high roughness scaffolds. Under these conditions cells preserved their morphology and structural properties. The system’s activation was revealed by ELISA experiments, by measuring the secretion of interleukins IL-2, IL-4 and specific antibody production in cell culture supernatants. T cell activation was demonstrated by detection of IL-2 and IL-4 in culture supernatants, while the development of antigen specific response was confirmed by detection of HSA-specific antibody production. The 3D micro and submicron laser textured transplantable scaffolds with tunable morphology and chemistry enable a multi parametric assessment of surface cues affecting macrophages activation and antigen specific response.
Structural and Elemental Analysis of the Bone-Implant Interface

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The mechanisms of osseointegration are not fully understood. It has been demonstrated that the gene expression pattern correlates with the mechanical stability at different titanium implants. However, the relationship between those findings and the interfacial structural composition has not been determined. The present study aimed to investigate the temporal changes in bone morphology and composition at machined and oxidized implants. The implants were retrieved en bloc from rat tibia at 6, 14 and 28d. Histology, histomorphometry and backscatter scanning electron microscopy (BS-SEM) analyses were performed. Ultra-thin sections were also prepared by focused ion beam (FIB) for transmission electron microscopy (TEM) analysis. At 6d, higher mineralized bone area (BA) was detected at oxidized implants. Over time, machined implants showed constant increase in BA whereas oxidized implants had a constant increase in bone contact (BC). BS-SEM, TEM and EDS analyses revealed mineralized tissue inside submicron pores and in intimate vicinity of the oxidized surface. The morphometric data supports previous gene expression data of higher bone remodeling at oxidized implants after 6d. Moreover, the morphometric data provides a structural correlate to previously reported biomechanical data, showing a significant increase of removal torque for oxidized implants over time. It is suggested that implant surface properties significantly influence the bone formation kinetics, which is determined by the regulation of cellular activities at the interface. This, in turn, influences the degree of bone mineralization and stability of the bone-implant interface.
Albumin Coated Allograft Increases Bone Healing: Cell Therapy without Exogenous Stem Cells

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Blood serum fractions such as platelet rich plasma or activated serum are known adjuvants in bone replacement therapies with an unclear mode of action. In previous experiments we have shown that serum albumin coating on bone allografts can significantly increase stem cell number in vitro, raising the possibility that albumin alone may be an effective proliferative agent in bone remodelling. In the present experiment, we investigated the bone formation by filling critical size defects with serum albumin coated allografts in rat cranial defect model. Bone formation was followed by computed tomography at 1, 3, 5, 7, 9, 11 weeks postoperatively. The area of the remaining defect and density were calculated. At five weeks significant difference was seen between the control groups and the albumin-coated group in the size of the remaining bone defect (no graft control 62.1 ± 7.8 %, uncoated graft 63.4 ± 12.8 %, albumin coated graft 15.2 ± 6.6 %). In the albumin-coated group every bone defect healed completely by the 9th week. In addition, from the 5th week the albumin-coated group showed significantly higher bone density values compared to both controls. By the 11th week the defects treated with the albumin-coated graft reached over 1000 Hounsfield unit, while controls remained below 700. In conclusion, the present investigation shows that implanting serum albumin coated allografts significantly reduce healing time in a critical size defect. These results may be explained by the idea that albumin coating provides a convenient milieu for stem cell function and proliferation, resulting in faster remodeling of the graft.

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Lectin-functionalized Silk Fibroin tailors Cytoadhesion on Biomedical Scaffolds

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Various tissue engineering (TE) approaches are based on silk fibroin (SF) as scaffold material because of its superior mechanical and biological properties compared to other materials. The translation of many one-step TE approaches to the clinical situation has failed so far due to the requirement of a prolonged cell seeding step before implantation. We propose that the plant lectin WGA (wheat germ agglutinin) covalently bound to SF, will mediate cell adhesion in a time frame acceptable to be part of a one-step surgical intervention. After establishment of a modification protocol utilizing carbodimide chemistry, we examined the attachment of ligament fibroblasts and adipose-derived stroma cells (ASC) on WGA-SF compared to pure native SF. After a limited time frame of 20 min the attachment of ASCs showed about a 17-fold increase as compared with that to pure native SF. Furthermore, we could demonstrate that the adhesion of ASCs on the WGA-SF does not influence proliferation behavior after 4 d of cultivation analyzed via BrdU-assays. To test the influence on differentiation, ASCs have been cultured in osteogenic medium on the SF-WGA (3 wks). Von Kossa staining and alkaline phosphatase assays verified the perpetuation of ASCs differentiation capacity. To test for in vitro immune response, peripheral blood mononuclear cells, did not show any altered proliferation behavior for 5 d in contact with the different silk groups.

The improved cell-adhesion without adverse effects on the proliferation and differentiation of ASCs as well as no detectable influence on the in vitro immune response suggests that the WGA-modification of SF offers important benefits for translation of SF scaffolds into clinical applications.

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Outgrowth Endothelial Cell-specific Capture by Oriented Immobilization of Antibody on Cobalt-chromium Alloy Surface

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The damage of endothelium caused by stent implantation is a critical factor for restenosis and thrombosis. The restoration of damaged endothelium can be a well-built way to reduce late stent thrombosis and restenosis. It has been reported that endothelial progenitor cell (EPC) can differentiate to endothelial cell and then facilitate re-endothelialization. Some groups have developed EPC capture strategies using antibody (Ab) immobilization technologies for the restoration of endothelium. We focused on CD146 as a target marker that is expressed on the surface of not EPC but outgrowth endothelial cell (OEC). Dopamine coating method was used to immobilize some Abs on metal surface. It is demonstrated that anti-CD146 Ab-immobilized stent can capture EPCs and restore a damaged endothelium. First, polydopamine (pDA) coating on cobalt-chromium (Co-Cr) surface was accomplished for the antibody immobilization. And, anti-CD146 Ab-immobilized Co-Cr was evaluated by immunofluorescence, FT-IR, ELISA, and water contact angle. In particular, anti-CD146 Ab-immobilized Co-Cr surfaces were examined by an OEC capturing assay. pDA coating decreased water contact angles of bare Co-Cr surface and thereafter CD146 Ab immobilization increased those of pDA-coated Co-Cr. In addition, other measurements qualitatively characterized CD146 Ab immobilized Co-Cr. ELISA assay determined the surface density of CD146 Ab of over 10 ng/cm2. OEC capturing assay indicated that CD146 Ab-immobilized Co-Cr showed significantly high adhesion rate of OEC as compared to others, bare and pDA-coated Co-Cr surfaces. The specificity for cells and Abs was also demonstrated.
On-Chip Study of Bioactive Agents Release from Arrays of Hydrogels Particles Using Patterned Superhydrophobic Surfaces

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Effective tissue regeneration is often dependent on prolonged and localized delivery of bioactive agents. This is usually determined by a correct tailoring of biomaterials used as biomolecules carriers. A considerable number and variety of platforms for the rapid and high-throughput study of biomaterials-cells interaction have been proposed. However, cells-biomolecules interactions studies in high-throughput approaches have been limited to direct contact tests, where the content of soluble biomolecules in time and space is neglected. In previous approaches, we proposed the use of superhydrophobic surfaces patterned with hydrophilic regions for the study of 3D biomaterials and their interactions with cells. Since conventional tests to determine biomolecules release rates from biomaterial matrixes are time and resource consuming, we proposed the adaptation of these chips to study the release of a growth-factor model from polymeric matrixes.

The chip based on wettability contrast was adapted with arrays of ring-shaped hydrophilic transparent regions, where a concentric superhydrophobic circle was kept. Protein-loaded hydrogel spheres containing different concentrations of alginate and protein were dropped in these repellent regions with minimum protein loss. The acquisition of sequential images of each spot using microscopy allowed monitoring protein release by image-based fluorescence quantification. The easy access to the medium insights the possibility of studying biomolecules release kinetics in changing conditions along time. Moreover, the platform is adaptable for future automation, in order to mimic standardized organs dynamics. We concluded that the chip shows applicability for rapid and efficient in vitro bioactive agents release studies.

Acknowledgments

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Design, Synthesis and Cellular Studies of a New Cell Adhesive Dimeric Peptide with Osteointegrative Properties

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Functionalization of metallic materials with cell adhesive molecules from the extracellular matrix (ECM) has extensively been used to improve cell-material interactions and enhance biointegration (e.g. osseointegration of bone implants). Classical strategies have focused on the use of full-length proteins from the ECM; however, their use bears several disadvantages that preclude their widespread use. Single peptide motifs are able to circumvent most limitations of natural proteins, but they often fail to recapitulate the protein’s full biological activity, in particular when the binding to multiple cell receptors is required. For this reason, the development of new coating molecules with the capacity to present distinct bioactive motifs has great potential for application in biomaterials. In this work, we present a synthetic approach to produce branched peptides presenting the RGD sequence and the α5β1-selective synergy domain PHSRN. These molecules, upon immobilization on titanium, enhanced the adhesion rate and spreading behavior of sarcoma osteogenic (SAOS-2) cells compared to control samples (plain Ti) or samples coated with mixtures of the single motifs RGD and PHSRN (1:1 ratio). Furthermore, these biomaterials stimulated the expression of typical osteoblastic markers. These peptidic structures might be useful to combine a number of cell adhesive molecules in order to promote cell-selective responses on biomaterials, and are thus promising candidates for applications in tissue engineering.
Biological Effects of RGD or SIKVAV Grafted Peptides: Different Strategies to Improve Bioactivity on PLLA to Guide Cellular Contractile Phenotype.

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Because of the high variability in mechanical, physical and chemical properties, biodegradable polymeric materials (i.e. Poly-L-lactide acid-PLLA) are primarily used for tissue engineering applications. In order to improve the material’s bioactivity, in this work, PLLA surface was modified by grafting RGD, a fibronectin derived adhesion motif, and SIKVAV, a laminin derived motif, and rat cardiac (H9C2) and mouse (C2C12) myoblasts proliferation and differentiation on modified PLLA were evaluated. A PLLA film was made by casting technique and in order to verify the surface modification, XPS analysis was performed. After seeding, cells viability and proliferation were confirmed by MTT assay and Proliferation Cell Nuclear Antigen (PCNA). Myf5, myogenin and myosin heavy chain (MHC), Western blot were used to analyze differentiation. MTT assay showed no significant differences in cell viability between cells cultured on different grafted surfaces. Western blot showed that cells cultured in presence of SIKVAV significantly increased differentiation markers expression. Results were confirmed by SEM technique, where cells cultured on SIKVAV grafted surfaces showed an enhanced spindle-shape morphology compared to cells cultured on other materials. These results suggest that bioactive molecules grafting could be useful on polymeric scaffolds for guiding cell phenotype expression, and, to ultimately maintain adequate biological characteristics suitable for functional tissue regeneration.
Peptide Conjugation on Polymers: RGD Presentation on Poly-caprolactone Surfaces in Complex Media at the Bio-interface

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Surface bioactivation through covalent insertion of adhesion peptides represents the current strategies to overcome the poor cytocompatibility providing additional features to synthetic materials. However when exposed to serum culture media, large protein adsorption occurs that mediate cell recognition, hindering the immobilized specific ligands. Here the presentation of RGD immobilized on polycaprolactone surfaces has been investigated at all level involved in the signal recognition starting from bio-conjugation, passing through protein adsorption to adhesion events at over time. To this aim, GYGRGDSP was grafted to aminolyzed PCL, then morphological and topological characteristics of surfaces were investigate through WCA and AFM. The presence of immobilized peptide was evidenced with more surface specific infrared spectroscopy by means PM-IIRRAS and the density by a colorimetric assay. The adsorption of serum proteins was assessed by QCMD and the viscoelastic properties of the proteinaceous layer were extracted from the analysis at different overtones. A microparticle based assay, by using an integrin mimicking binding peptide, was developed as a tool to investigate, in a “cell-free” fashion, the presentation of RGD in complex environments (in presence of FBS) and to measure the efficiency of ligand presentation. Biological response in different contexts (serum added and serum free) was assessed through the analytical evaluation of cytoskeleton and focal complexes size and organization over time.
RGD-modified Poly(ε-lysine) Dendron Functionalization of 3D Printed Scaffolds and Human Mesenchymal Stem Cell Response

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Development of new smart scaffolds for guided tissue regeneration is still a main goal in tissue engineering. In this work, polylactic acid (PLA) and PLA/CaP glass biodegradable scaffolds were fabricated by additive manufacturing and functionalized with RGD-dendrons to improve their bioactivity. The aim was to develop and characterize dendron-functionalized PLA based scaffolds.

Scaffolds fabricated by a 3D printing system were functionalized with RGD-modified poly (ε-lysine) dendron (RG1K(VPGVG)₆G2G3K (RGD)₁₆) assembled by solid-phase synthesis. The dendron was synthesized and characterized. Surface functionalization (NaOH+EDC/NHS activation+immersion in dendron solution) of the 3D structures was achieved. Adhesion, proliferation and morphology of hMSCs were evaluated by LDH activity and immunofluorescence.

Successful synthesis of the dendrons was confirmed by HPLC and mass spectroscopy. SEM, FTIR and ninhydrin staining revealed the presence of amide groups typical of peptide-based dendrons on the functionalized scaffolds. PLA scaffolds seemed to be better coated with the dendron than the composite ones. Cells seeded on non-functionalized materials showed mostly fibroblastic morphology, while cell aggregates, forming spheroids, were seen on the functionalized scaffolds. Moreover, β-integrin staining was more evident for cells seeded onto functionalized scaffolds. LDH assay showed a greater number of adherent cells on functionalized PLA/glass scaffolds.

3D scaffolds were successfully functionalized with RGD-dendron showing a positive biological response. The potential of the functionalized scaffolds to form active cell spheroids present promising options for tissue regeneration applications.
Quantitative Characterization of Adhesion and Cytomechanics of Single Cells and their Interactions with Biomaterials by AFM

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Topography and mechanical properties of biomaterials are crucial parameters influencing morphology, adhesion, and mechanics as well as the development of cells. Atomic force microscopy is a powerful tool not only to study the morphology in terms of high resolution imaging and roughness measurements, but also to map mechanical and adhesive properties of the cells and tissues. Combining these remarkable abilities with optical microscopy allows for extensive characterization of biomaterials. We developed a new force curve based AFM mode - Quantitative Imaging (QI™) for a faster and better access to soft, sticky, or fragile samples avoiding lateral sample displacement. We investigated topography, adhesion properties, and local distributed Young’s modulus of living cells on related biomaterials. A specialized platform - CellHesion® - has been developed to run single cell force spectroscopy (SCFS, see Fig.1) with a need for long-range cell-surface binding experiments with up to 100 microns pulling length. SCFS quantified the cell adhesion force and the contribution of different components, e.g. from the extra cellular matrix to implant materials (e.g. cochlear implants), as well as cell-cell adhesion. A new solution will be demonstrated to transfer attached cells through the liquid-air interface. This approach allows measuring the adhesion of the same single cell on different materials. We present a strategy to comprehensively characterize biomaterials as well as their interaction with cells and influence on cell behavior. Nano-mechanical analysis of cells increasingly gains in importance in different fields in cell biology like developmental biology and cancer research.

Fig. 1: Sketch of a SCFS experiment. The probe cell is approached to (1) and pressed against the substrate (2) with a defined Setpoint force (F) for a defined time (t). When the cell is separated from the sample (3) interactions like maximum adhesion force (Fmax) and single unbinding events (force jumps (J) and those that are preceded by membrane tethers (T)) are visible in the force distance curve. The contact part of the Approach curve allows for applying elasticity models (E).
Chondrogenic Differentiation of Progenitor Cells on Bioactive Peptide Nanofibers

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Healing of cartilage defects is an important medical problem since the current treatment methods are ineffective to restore full function and return the tissue to its healthy state. Having a slower metabolism than other tissues, cartilage tissue cannot fully repair itself after damage. For this reason, developing therapies for the treatment of cartilage tissue damages that occur as a result of common joint diseases like osteoarthritis, rheumatoid arthritis and accidents, is of major importance. Regeneration of damaged cartilage tissue and complete recovery of its functionality may be possible with regenerative medicine studies, which hold great promise by offering novel solutions for generation of functional tissue substitutes. Glycosaminoglycan (GAG) molecules are important constituents of both developing and mature cartilage extracellular matrix (ECM). Several studies indicate that actions of regulator proteins of cartilage development depend on these GAGs. In this study, we explored the role of GAG mimetic self-assembling nanofibers as a scaffold in inducing chondrogenic differentiation of chondroprogenitor ATDC5 cells and mouse mesenchymal stem cells. Chondrogenic differentiation was indexed by sulfated GAG deposition and expression of cartilaginous ECM proteins such as collagen II and aggrecan. Moreover expressions of genes specific to cartilage tissue were investigated to reveal the effect of GAG mimetic peptide nanofibers on chondrogenic differentiation.
Control of Mesenchymal Stromal Cell Colonization Using Synthetic Micro-
Niches Engineered Via Two-photon Laser Polymerization

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Cell therapies using autologous MSC are approaching clinical implementation, provided that
adequate culture techniques are established. Within this context, using two-photon laser
polymerization we have developed an advanced culture substrate for MSC, consisting of
rapid-prototyped 3-D micro-niches, engineered on flat surfaces. In previous work, we have
optimized the fabrication technology and the niche geometry. Here, we optimize the niche
layout. We used the SZ2080 photoresist and the Irgacure 369 photoinitiator to write sets of 3-
D micro-niches on standard cover glasses. Each niche was a prismatic confinement grid,
90x90 μm in side, 45 μm high, with an internal lattice of open pores, of size graded from 10 to
30 μm. On each cover glass, 7 niches were arranged in hexagonal patterns of varying side.
Bone marrow-isolated rat MSC were seeded at a density of 20 kcells/cm², and cultured up to
3 weeks. We analyzed cell density (dapi), proliferation (Ki67), and differentiation (histology,
IF for Collagen types I and II, and osteocalcin). Cells formed spontaneous colonies, of
average cell density 170 kcells/cm², randomly distributed with average relative distance 300
μm on the flat surface, while centered in the niches on the niche-patterned surface. At
prolonged culture, the colonies became spherical cell aggregates, of average diameter 90
μm, in which the extra-cellular matrix stained positive for cartilage markers. Our evidence
suggests that the engineered niches are able to guide the spontaneous tendency of MSC to
colonize and form aggregates of differentiated cells in culture. We now plan to niche-pattern
a wider surface, to extract quantitative data to consolidate our findings.
3D Micropatterned Silicon Structures Influence Neuronal Cell Response

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Controlling the outgrowth of neuronal cells is of critical importance in a wide spectrum of neuroscience applications. However, the study of neuron cell outgrowth on more complex topographies remains limited. Phenotype alteration of neuronal cells cultured on traditional flat substrates lacking structural cues, emphasize the necessity to shift from 2D to 3D or multi-scale cell culture models. The aim of the present study was to investigate the cellular response to the topographical cues. 3D micro/nano structured silicon surfaces used were fabricated using a femtosecond pulsed laser. Variation of the laser fluence alters the surface morphology, leading to a rippled-type or a conical spiked type morphology. As a result, the respective patterned substrates exhibit different geometrical characteristics and wettabilities. Established cell lines or primary cells with nerve cell phenotype were cultured on such substrates. More specifically, PC12 cell cultures in the presence of nerve growth factor showed that, contrary to low and intermediate rough patterned surfaces, highly rough ones exhibiting large distances between the microcones did not support PC12 cell differentiation, independently of the chemical coating. The experiments with primary DRG/SCG cells showed a good attachment, outgrowth and network formation on all the surfaces. Remarkably, there was a differential orientation of the cells on the various substrates. In particular, cells were randomly oriented on low roughness surfaces, whereas there was a trend for parallel alignment on the intermediate and high roughness substrates. Our results suggest that the geometrical characteristics of 3D micro/nano structured Si surfaces alone can influence specific cellular functions.
Fabrication of Artificial Extracellular Matrix by Electrodrop Casting of Graphene Oxide on Titanium for Osteogenic Differentiation

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Extracellular matrix (ECM) is a very useful tool in regenerative medicine. However, manipulation of ECM property is still challenging and thus fabrication of artificial ECM is a promising approach. Recent studies report the efficient osteogenic differentiation of mesenchymal stem cells using graphene oxide (GO). In this study, we present the fabrication of GO coated Ti ("Tigra") as an artificial ECM by using electrodropping system and the evaluation of its osteogenic potential with MC3T3-E1 preosteoblasts. The dual syringe system is employed for the fabrication of Tigra followed by surface functionalization with fibronectin (FN-Tigra) for artificial ECM. The morphology, topography, and mechanical properties are also examined using bio-AFM, SEM, force spectroscopy, Raman spectroscopy, and XRD. Osteogenic differentiation of preosteoblasts was investigated by measuring alkaline phosphate (ALP) activity, calcium content, van Kossa/Alizarin red staining, and immunofluorescence. Tigra was effectively functionalized with FN to enhance the cell adhesion. The preosteoblasts maintained higher cell viability on the FN-Tigra surface. Furthermore FN-Tigra can promote the osteogenesis of preosteoblasts compared with bare-Ti. Calcium content and ALP activity was much better with FN-Tigra than Ti. Current work suggests that artificial ECM can be established on the Ti surface and should be useful in biomedical applications.
Use Disulfide-bonded Biopolymer as Detachable Matrix to Culture Periodontal Ligament Fibroblast Cell Sheet for Periodontitis Treatment

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In last decade, Okano et.al. proposed a new way to engineer cell sheet, and the cell-sheet engineering can retain extracellular matrix for tissue engineering has been a good clinical application approach. We incorporate the idea by Akashi et al. which utilize disulfide bonds in forming the porous hydrogel, and further developed a system using biopolymer as base and disulfide bonds cleavage as the critical point of cell sheet detach system. The staining results of methylene blue, which binds to the amine group of γ-PGA, shows that the two-stage reaction is uniform with good integrity. In addition, the ESCA analysis show the element changes at every response as expected, respectively. This further confirms that these membranes surface were successfully modified and by adding reducing agent, the disulfide bonds can be easily cleaved. Cytotoxicity tests show good biocompatibility of the matrix PVDF membranes. Finally, the cell sheet detachment was successfully done by adding Cysteine after NIH3T3 or PDL (Periodontal Ligament) fibroblast culture for a week. In summary, Nitrogen plasma treatment following adding Cystine, EDC, and γ-PGA generates well formed two-stage surface-modified PVDF membranes. PVDF membranes seem to be a novel and potential material in engineering cell sheet and could be useful in harvesting cell sheets. This system is expected to bring much higher cell adhesion and cell activity fulfilling the needs for periodontitis treatment.
Biocompatibility and Cell Guidance on Photo-Patterned Azopolymers Films

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Cell materials interaction studies continuously demonstrate the relevance surface patterning in controlling cell behaviour. Indeed, cells may respond differently so producing different response in terms of adhesion and growth as a function of the surrounding topography which provides to cells a wide variety of physical signals at the scale of tens of nanometers to micrometers. Here, we investigate the response of human mesenchymal stem cells (hMSC) onto new azo-polymers photo-patterned in single stripe or multi-grooves via two-photon absorption technique to validate their use as candidate in the biomaterials field. Atomic Force Microscopy (AFM) was used to analyze the topographical features of produced the patterned surface. hMSC cells were cultured in (α-MEM) medium supplemented with 10% fetal bovine serum, antibiotic solution (i.e. streptomycin 100 μg mL\textsuperscript{-1} and penicillin 100 UmL\textsuperscript{-1}), until 6 days on films with different patterns. Cell response was evaluated by confocal and optical microscopy in terms of adhesion and viability.

Azopolymer films show two different patterns investigated by AFM: a) a single scribe – about 150 μm thick - and b) a multi-groove area with a 3500 nm period grating. hMSC cells adhere and proliferate on azopolymers films independently on the imprinted pattern. Only a slight increase of proliferation is observed onto the multi-grooves which may be correlated to the higher ability of cells to recognize surfaces with higher structural complexity. This is also corroborated by the optical analysis of the body cells morphology, after 24 hours of culture, which show an evident tendency to form clusters in the case of surrounding multi-groove area.
Strategy for Surface Functionalization of PEEK-Based Materials for Prosthetic Uses

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Nowadays, the surface functionalization of materials to be used in prosthetic devices is a high desirable approach. In this work, a suitable method to obtain biocompatible materials has been presented with the challenge of working with a very chemically inert polymer: Polyetheretherketone (PEEK). The latter is a thermoplastic with acceptable biocompatibility and has been used in the manufacturing of implants, prosthetic devices and cell culture matrices. However, this kind of polymers shows poor tissue integration properties. For this reason, it is highly desirable to develop methods to modify its surface for medical applications while preserving their excellent mechanical, chemical and structural properties of the bulk. The technique used for the PEEK surface functionalization was based on wet-chemistry. On the whole, this work reports chemically functionalized PEEK polymers with bio-orthogonal groups like azido, terminal alkyne, cyclooctyne, maleimido, thiol, suitable for bioconjugation with light-responsive agents (e.g. dansyl group). It showed four different routes based on “click” chemistry advantages, such as mild conditions, high selectivity and high yields. Indeed, organic reactions carried out at the solid-liquid interface restrict the changes to specific surface groups and do not alter the structure of the polymer bulk and its physico-chemical properties. The resulting materials were characterized by XPS, AFM and fluorescence methods in order to to validate the proposed method. The results confirmed that the latter indeed modified the surface while preserving the PEEK bulk properties. These promising results revealed that this is a suitable method that could be used for obtaining PEEK materials with better tissue integration properties (e.g. osseointegration
Titanium (Ti) and its alloys have the ability to strongly integrate with bone tissues because of their unique surface properties. Surface modifications to Ti surfaces have been shown to alter *in vitro* and *in vivo* behaviour and performance; this could be due to increased cell interaction and adhesion. To better understand how cells adhere to modified Ti surfaces, we investigated the attachment of individual human mesenchymal stromal cells (MSC) to different biomaterials substrates (TCP, TiO$_2$ coated glass, Ti smooth (SMO) and Ti rough (SLA) surfaces using atomic force microscopy (AFM). Silicon nitride AFM cantilevers were fixed with Con A coated glass beads as described by (Wojcikiewicz et al., 2004), and then attached to individual MSCs. Measurements of the cantilever deflection during this process allowed the forces between the cell and surface to be quantified in the form of “force-distance curves”. Mechanical parameters such as full force of detachment, the displacement needed to completely remove the cell from the substrate, and the work of detachment were observed from these force curves. Essential parameters that have an impact on cell attachment, such as force of contact, contact time and cell movement velocity, can be monitored. The force curves show significant changes to MSCs’ adhesive forces on different modified substrates at fixed time points. The MSCs adhesion forces changed significantly between the modified substrates. It was possible to determine the maximum force of cell attachment on all the surfaces.
Biocompatible Medical Devices: Modification of Polymer and Metal Surfaces

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One major problem with medical devices is the fact that surrounding tissue is not able to recognize the surface as tissue like material. In case of blood oxygenators, this can induce blood coagulation which leads to blocking of the surface and the gas exchange. For metal implants such as pacemakers, incomplete tissue integration frequently promotes infection and leads to implant migration.

To prevent these problems, we are going to modify and humanize the surface. One approach is to covalently attach RGD-peptides to the implant surface so that cells can grow on it and the device is able to integrate in the tissue. The RGD-modified blood oxygenator can be seeded with lung endothelia cells which stealth the surface from the blood stream.

In a first step the activation of surfaces is performed with plasma. This technique enables the chemical derivatization of surfaces in increasing wettability or active groups. Via spacer it is possible to bind an oxanorbornadiene to the surface. With this system a ligation of the azide-labelled RGD-peptide or an active component is performed in a copper-free “Click”-reaction.

The by plasma activated surface can also be modified with quaternary ammonia salts. They are known to be antibacterial. This approach can prevent biofilm formation on implants.
Nanostructured Polymeric Coatings Based on Chitosan and Dopamine-
modified
Hyaluronic Acid for Biomedical Applications

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In a marine environment, specific proteins are secreted by mussels and used as a biogluce to stick to a surface allowing generate irreversible bonding. Adhesive secreted proteins of mussels present an unusual amino acid 3,4-dihydroxyphenylalanine (DOPA). The outstanding adhesive properties of these materials in the harsh conditions of the sea have been attributed to the presence of the catechol groups present in DOPA. Inspired by the structure and composition of adhesive proteins in mussels, we used dopamine-modified hyaluronic acid (HA-DN) prepared by carbodiimide chemistry to form thin and surface-adherent dopamine films. This conjugate was characterized by distinct techniques, such as nuclear magnetic resonance (NMR) and ultra-violet spectrophotometry (UV). Thereafter, multilayer films were developed based on Chitosan (CHT) and HA-DN using the Layer-by-Layer (LbL) method. The formation of these films was investigated in-situ by quartz crystal microbalance with dissipation monitoring (QCM-D). The adhesion properties of the coatings were also analyzed. In vitro tests using two distinct cell sources showed an enhanced cell adhesion for the biomimetic films that contain catechol groups, demonstrating their potential to be used in biomedical applications.
Metalloenzyme Inspired Peptide Nanofibers for Bone Mineralization

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Designing enzyme inspired synthetic systems through artificial construction of active sites of protein structure and function has potential use in biomedical and industrial applications. Novel strategies based on amino acids or peptides as characteristic molecular moieties have resulted in a remarkable expansion of the field of enzyme mimics. Similar to peptide chains that form active sites in enzymes, synthetic peptide systems aim to preserve the function of enzymes in a simpler and more stable system and stability. In this study, self-assembled peptide nanofibers were developed to mimic natural enzyme activity. Alkaline phosphatase (ALP) has a catalytic site mainly composed of histidine residue and zinc ion to produce inorganic phosphate under alkaline conditions. We designed self-assembling peptide amphiphile (PA) molecules, which contain histidine residues in different positions as Zn²⁺ coordination site on the peptide nanofiber system. These peptide nanofibers were used in inorganic calcium phosphate formation where the enzyme mimicking nanofibers produced inorganic phosphate ion from organic phosphate molecules. The use of this system to enhance biomineralization was investigated by in vitro experiments by using SAOS-2 cells. Cells cultured on the enzyme mimicking nanofiber networks displayed bone nodule formation within three days in the absence of osteogenic differentiation enhancing factors such as dexamethasone and ascorbic acid. These results show that peptide nanofiber systems can be used as efficient platforms for mimicking enzyme activities.
The Effect of Electric Current on the Cell Adhesion Forces Quantified by Fluidic Force Microscopy

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When an external electric field is applied to cultured cells, the local pH changes in the close vicinity of the electrode and the cell-surface adhesion becomes weaker. Changes in the cell adhesion can be qualitatively observed with optical microscopy but to obtain quantitative information, we used fluidic force microscopy (FluidFM). FluidFM is based on atomic force microscope using hollow cantilevers connected to a fluidic circuit. FluidFM enables picking up individual cells by applying under-pressure while measuring the occurring adhesion forces.

Cells were cultured on indium tin oxide or gold electrodes and after the cells were adhered, positive or negative electric current pulses of different magnitudes were applied and the adhesion force between the cell and the surface was measured. The applied electric current remarkably lowered the cell-surface adhesion forces which depended both on the magnitude and on the polarity of the applied currents.

Controlling cell adhesion with electric currents can provide a tool for patterning and separating cells, and we have shown that the effect of current on cells can be quantified by using FluidFM.
Ion Beam Effects on Chitosan Membranes for Tissue Engineering Applications

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Chitosan has been studied for various practical applications because of functions such as biodegradability, low toxicity, and acceleration of fibroblast formation in animal body, acceleration blood clotting, drug delivery, antimicrobial activity and high solubility in water. In this study, we attempted to prepare and characterize the chitosan membrane surfaces induced by metal–gas (MEVVA) ion implantation. Chitosan membranes were prepared in two different microstructures to investigate the structure effects on the protein adsorption and in vitro degradation. Dense and asymmetric chitosan membranes prepared by dissolving in acetic acid solution. For dense membrane production, solvent casting method was used. For asymmetric membrane preparation dry/wet phase separation method was used by using 20 minutes pre treatment time. By changing this time pore size and thickness of the membrane is changed that also effects the membrane properties like diffusion ratio, water absorption, degradation time etc. Chitosan membranes then were implanted by C and C+N ions by using MEVVA ion implanter. As a result of these, we investigated the effect of ion implantation on the protein adsorption behavior, in vitro degradation and cell attachment properties of chitosan films before and after the ion implantation. The chitosan films were prepared by solvent casting method for dense films, and dry/wet phase separation method is used to obtain asymmetric chitosan membranes. Characterization studies of these membranes were performed by using Scanning Electron Microscopy (SEM), Fourier Transform Infrared Spectroscopy (FTIR), and Differential Scanning Calorimetry (DSC). The ion implantation effect on ion beam modified chitosan membranes for neural cell attachment (B35) is also examined for different dose and energy parameters of the beam. As a result of that, surface modification by ion implantation with $10^{16}$ cm$^{-2}$ dose, and 1 pps frequency, 20 kV acceleration voltage were the most appropriate values in order to neural cell attachment and neurite extension capability with chitosan membranes.

\textbf{Keywords:} Chitosan, protein adsorption, biodegradation, ion implantation
ECM Mimetic Nanostructured Titania Surface for Osteogenic Cell Fate

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Titanium and its alloys are important dental and orthopedic implant materials due to their superior characteristics in terms of corrosion resistance, bioinertness, and mechanical stability. However, osseointegration of these implants is of major concern since improper integration of the implant to the living tissue might end in rejection of the implant in the long term. Establishing integration of native bone tissue to the implant material is a challenging task requiring various factors. Since the surface of the material makes the first contact with the host tissue, altering surface parameters such as its chemistry, hydrophilicity, and topographical features have been intensively studied. In this study, we developed a novel method to modify titanium surfaces to create a biofriendly surface texture for osteoblasts, an important bone cell type for osseointegration. Peptide nanostructures coated on the surface of titania were exploited as a template and further nanostructured TiO₂ fabrication was performed to form a nanofibrous titania scaffold, which bears structural resemblance to the native extracellular matrix of bone tissue. Peptide nanofibers (6-7 nm in diameter) were successfully coated with TiO₂ by using atomic layer deposition technique. Conformity and uniformity of TiO₂ were found to be excellent and unmatched by any previously reported method. This scaffold was used to grow osteoblast-like SaOs2 cells to study the adhesion of cells and to induce osteogenic differentiation. Our results demonstrated that osteoblast-like cells adhered onto surface and spread into their native morphology. Their differentiation efficiencies are under investigation. Through mimicking the nanofibrous texture of the ECM on the implant surfaces, we aim to enhance osseointegration capability of these surfaces, which in turn would increase therapeutic efficiency of the implants in both short and long term after implantation.
Protein Binding Functionalisation Of Plasma-Derivatized Silicone Surfaces

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Silicone is a versatile material for medical applications, but is essentially bio-inert. The ability to bind proteins to the surface allows functionalisation & may benefit wound healing and other biocompatible medical device applications. This study characterized protein binding to oxygen-plasma treated silicone surfaces (O2) or further derivatised by plasma polymerisation with acrylic acid (AcA) (Altrika). Non-blocked polystyrene wells (PS) were used as reference. Discs of silicone sheets with fibrinogen (FBG) (5-250 µg/ml) as a test protein were incubated in pre-blocked ELISA wells. In some cases, FBG was crossed linked with 2% glutaraldehyde (GTA). Bound FBG was detected using an ELISA method. Total protein was assayed by Bradford Ultra reagent (Novexin). Optimal primary and secondary antibody concentrations were determined and FBG binding affinity and capacity to PS, O2  & AcA surfaces determined. FBG-silicone binding curves were characterized by saturation at around 150 µg/ml to all types, with the highest binding to AcA. Binding for PS samples saturated at around 50 µg/ml. GTA cross linked FBG was detected by a polyclonal anti-fibrinogen antibody. From total protein measurements approximate values for FBG binding were 1.27 µg/mm² (all of the silicone surfaces); 0.74 µg/mm² (PS). Release of protein by trypsinisation and slot-blot assay provided an alternative estimate of total bound protein. Silicone surfaces show 2x protein binding capacity than PS but with lower affinity. Bound protein was not removed from either surface, but binding could be reduced by co-incubating with non-ionic surfactant. While improved derivatisation is needed to increase surface binding capacity, this method demonstrates feasibility.
Ion Implantation and Plasma Deposition for the Fabrication of Patterned Nerve Conduits

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In this work, we explored the suitability of various kinds of ion implantation and of plasma deposition for the selective, enhanced growth of neuronal cells on substrates that could be applied to human surgical/medical needs for neural regeneration. As necessary components of the overall research, we explored several other related fundamental and applied issues, including a quantitative survey of the suitability of specific plasma/ion-based surface modification parameters on biological aspects, and the fabrication of large neural arrays or in experimentally designed patterns which allows to combinatorial approaches with 2D lithographical applications. As a result of this, we fabricated patterned neural conduits with biodegradable polymers. Our research will thus have both fundamental and applied aspects, and it has broad impact to medical technology as well as to several other subfields of bioelectronics, plasma and ion beam physics, materials science, and ion beam biotechnology.

Figure 1. Au ion implanted Polycaprolactame conduits for nerve regeneration in peripheral nervous system.

**Keywords:** Biodegradable Polymers, Nerve Conduits, Ion Implantation
Surface Functionalization of Biomaterials with Mucin Films

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Mucins are the main component of viscoelastic mucus gels that are known to protect the underlying epithelial tissues by providing hydration, physical barrier, and lubricity. In addition to their important biological functions, or to make use of these functionalities, mucin and mucus gels have been studied as coatings to functionalize surfaces of biomaterials. This idea is based on an important assumption that since mucin/mucus represent outmost layer of epithelium, biomaterials coated with mucins may be recognized as “self” rather than “foreign” bodies by the human immune system. In this study, we demonstrate that mucin films formed on surfaces of implants or biomedical devices display effective resistance to nonspecific adsorption of plasma proteins, i.e. antifouling properties. The efficacy of antifouling properties was observed to be varying depending on the composition of mucin films; in general, the composite films constructed from mucin and albumin was superior to those composed of purified mucin alone, which indicates a synergistic effect between mucin and albumin for antifouling properties. This observation also dictates the direction towards more effective formation of mucin films for biomaterials. Finally, composition-dependent antifouling properties of mucin films may shed new light on understanding other biocompatibility properties revealed by mucin films, such as the resistance to bacterial and viral adhesion, and favorable immune cell responses.
Surface Modification for the Improved Material Thromboresistance

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Cell-biomaterial interaction occurs at the interface of the implanted material at the molecular scale. Ions, single molecules and proteins govern cells interaction with material surface and regulate biocompatibility of the artificial material with host tissue. Varieties of approaches have been applied to improve the surface biocompatibility and particularly haemocompatibility of materials. In this work we investigated different nanoscale techniques to improve biocompatibility and thromboresistance of blood contacting materials for the medical devices such as artificial heart valves, cardiopulmonary by-pass circuits, vascular grafts and stents. Three main approaches to surface modification were exploited including: i) coating of the surfaces with inorganic oxides (TiO, TiON, ZrO), ii) immobilisation of the NO donors which can release NO from the material surface and iii) formation of nanocomposites with catalytic properties to generate NO when modified surface get into contact with the in vivo environment. The proposed mechanism of catalytic NO generation comprises a fast denitrosylation of the endogenous RSNO’s by catalytic properties of the transition metals. The physicochemical analysis of the surface coatings and blood cells interaction with newly modified materials were examined. In conclusion, we found that TiO, TiON and ZrO coatings improved stainless steel biocompatibility with good correlation between in vitro and in vivo data. Immobilisation of the NO donors on the surface was confirmed and synthesis of nanocomposites on the biomaterial surface with the catalytic activity to generate NO has been demonstrated.

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“Cell Response on Mineralized Self-assembling Peptides on Top of a Hybrid Scaffold”

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In this study we focus on the following strategy for “scaffold-on-scaffold” biofabrication: a combination of primary laser-fabricated scaffolds with attached secondary peptide scaffolds that could be used as a support for the directed growth of cells into biomineralized units. More specifically, we fabricated primary scaffolds by direct femtosecond laser writing of an amine containing photosensitive hybrid material. We then decorate these scaffolds with a gold monolayer which immobilizes novel self-assembled peptides. These peptides were designed based on a natural octapeptide scaffold further modified to contain acidic amino acids, in order to specifically promote the deposition of calcium phosphates. We further investigated the suitability of these peptides to support cell attachment and proliferation. More specifically, we evaluate the biological response of pre-osteoblastic cells on the functionalized scaffolds. Our results show that the designed peptide immobilized on a hybrid structurable material strongly supports a proliferation increase after 3 and 7 days in culture and exhibits a statistically significant increase of biomineralization. We propose that this strategy can be used as a ‘scaffold on scaffold approach’ for hard tissue regeneration.
Sulfonic Layer-by-Layer Nanocoatings Made of Marine-Origin Polysaccharides as Models for Cell-GAGs-like Interactions Studies

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Most of the human body polysaccharides (Ps) are sulfated (sGAG) and non-sulfated glycosaminoglycans (GAGs), linked to core proteins in its majority, forming proteoglycans. GAGs vary on the sulfation degree, sulfonic group (Sg) position and base units. sGAGs have very high growth factors (GFs) affinity through the Sg, participating, e.g., in cell proliferation and differentiation. Non-sulfated ones are also crucial and may have similar functions by cell marker binding. In fact, several studies report that GAGs influence cell behaviour, which however is not fully understood. Actual models make difficult the transposition to 3D systems, where both GAGs and mechanical stability are needed. Layer-by-Layer (LbL) is a very versatile technique to produce nanostructured coatings over any 2D/3D structure and was herein applied to develop 2D models to study cell-GAGs-like interactions, using marine-origin Ps, chitosan (CHI) and carrageenans (CAR), which have equivalent functional groups to the ones found in GAGs: -COOH, -NH₂ and -SO₃H. CHI and κ, ι and λ CAR (1, 2 and 3 Sg, respectively) LbL assembling was characterized by QCM-D, modelled by Voigt model, AFM, SEM, XPS and the effect over SaOs-2 cell line biomineralization investigated. ALP activity and biomineralization of SaOs-2 was improved in the presence of the coatings, being significantly higher on ι CAR coatings - Fig.1, indicating CAR have functions much more than attracting medium ions. CAR may be very reactive to SaOs-2 secretome and capture GFs, improving biomineralization. These models may be very interesting to study cell-materials interactions and easily brought over 3D scaffolds for tissue engineering applications.

Fig.1. Alizarin Red, SEM micrographs (bottom).
Evaluation of Different Surface Treatments to Enhance Attachment of Bioactive Molecules Mixtures for Cardiovascular Implants

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The attachment of bioactive molecules on CoCr stent surfaces is a very promising strategy to modulate endothelial cells (EC) response in order to recover artery structure and function. However, the efficiency of the surface treatments in order to attach the maximum bioactive sequences is very low. Moreover, the combination or the appropriate spatial presentation of these molecules remains to be elucidated. CoCr alloy (ASTM F90) discs were treated with plasma (PL) and etched with 5M NaOH 2h (NaOH) previous to silanization with 3-chloropropyltriethoxysilane (CPTES) and functionalized with RGD and REDV elastin-like polymers. SEM, interferometry, contact angle, zeta potential and XPS were used to determine physico-chemical surface properties at each procedure step. Coating stability tests after sonication or autoclaving were evaluated by XPS and cell adhesion. HUVEC cell studies were performed by means of fluorescent spectroscopy and microscopy. XPS confirmed the immobilization of biofunctional molecules on CoCr surfaces and determined 30-50% of covalently-attached biomolecules for silanized samples. All surfaces presented a decrease of attached bioactive molecules after sonication or autoclaving and nearly no differences were detected between chemisorbed and physisorbed series. This can be explained by the low efficiency of the silanization treatment and the denaturation of the biomolecule due to the stability treatments. The use of combined RGD and REDV polymers enhanced significatively HUVEC adhesion response.
Relationship Between Modified Surface of Biomaterials via Atomic Layer Deposition (ALD) and Endothelial Cell Growth

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Biomaterials can be broadly divided into the polymer, metal and ceramic, which have much different physical characteristics. Most of metal biomaterials have high mechanical strength and good wear resistance but lack of biocompatibility comparing to polymers. The good biocompatibility would be one of the important properties of biomaterials. In this study, we used the samples which were coated with a layer of zirconium dioxide thin film on the 316 stainless steel substrate by Atomic Layer Deposition (ALD) method. Then we seeded Bovine Aortic Endothelial Cell (BAEC) on the prepared materials mentioned above and observed the samples under cell culture condition to test the biocompatibility, we found that the oxide layer film can improve the biocompatibility. Moreover, we further used three types of dioxide layer (TiO₂, ZrO₂, HfO₂) with ALD and performed the cell tests with different thickness and roughness of the samples.

First we cultured BAECs for 24 hours to observe the morphology and adhesion of cells; then by MTT assay method, we indirectly compare the cell viability. Bovine aortic endothelial cell (BAEC), the cell type was used in this study. Vascular endothelial cells line the entire circulatory system from heart to the smallest capillaries in humans. They are essential in maintaining blood fluidity, and improving thromboresistance in the applications of many cardiovascular implants such as stents, vascular grafts, and heart valves. Therefore, culturing endothelial cells on the modified surface of biomaterials is the most important motivation of this study.

The experimental results show that the plated oxide layers can promote cell growth, particularly the titanium dioxide layer affect mostly pronounced, titanium dioxide might be able to have the potential for its application as vascular biomaterials.
Volume-stable Constructs Made from Porous Scaffolds and Fibrin Hydrogels for Adipose Tissue Engineering \textit{In vitro} and \textit{In vivo}

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Adipose tissue engineering is a promising approach for the treatment of soft tissue defects. One major challenge for clinical applicability is the maintenance of volume and shape of engineered adipose constructs. Therefore, in this study, composite constructs consisting of biodegradable porous polyurethane (PU) scaffolds as support structure and two different fibrin hydrogels as cell carriers were investigated \textit{in vitro} and \textit{in vivo}. The fibrin/PU-constructs were seeded with human adipose-derived stem cells (ASC) and displayed pronounced triglyceride accumulation and strong adipogenic marker gene expression (PPAR\textsubscript{\gamma}, C/EBP\textsubscript{\alpha}, aP2, GLUT-4 (qRT-PCR); leptin (ELISA)) after adipogenic induction \textit{in vitro}, in contrast to non-induced controls. By employing a stable fibrin gel developed by our group [1], fibrin/PU constructs with superior volume and size maintenance were obtained, as compared to a commercially available fibrin gel. An \textit{in vivo} study was conducted in immunodeficient mice. After 7 days of adipogenic precultivation \textit{in vitro}, the stable fibrin/PU constructs seeded with ASC were subcutaneously implanted either with or without running an arteriovenous bundle through the constructs. After 5 weeks \textit{in vivo}, well maintained construct volume and shape was observed. Histological analysis revealed many large patches of mature adipose tissue within the pores of the PU scaffold in constructs that had received a vascular pedicle. Apparently, the fibrin gel was replaced by coherent tissue perfused by a network of capillaries. Utilizing the composite constructs, further experiments regarding the origin of the developed tissue are currently under way.
Peripheral Blood-Derived Endothelial Progenitor Cells For Vascularized Bone Tissue Engineering

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Implantation of autologous progenitor cells has proven to be effective for enhanced bone tissue engineered graft success. Here, we examined 3 autologous endothelial cell sources in combination with mesenchymal stem cells (MSCs) in vitro and in vivo. Endothelial progenitor cells (EPCs) were isolated from New Zealand rabbit peripheral blood and bone marrow, and vascular endothelial cells (VECs) from mesenteric arteries. MSCs were isolated from rabbit bone marrow. RT-PCR was employed to examine gene expression of key vascular and osteogenic markers in all cell types, as well as in co-culture with MSCs (1:1) after 7 days. PB-EPCs and MSCs were co-cultured (1:1) on optimally-porous PLGA scaffolds for 2 days, then implanted in a rabbit ulnar bone defect (n=6) for 12 weeks. Constructs not seeded with any cells prior to implantation served as control. MicroCT was used to quantify regenerated bone mass.

PB-EPCs demonstrated significantly higher levels of BMP4, VEGF-R2 and thrombomodulin (THRMB), and similar levels of BMP2, collagen I (COL1), and VEGF compared to VECs. BM-EPCs displayed significantly lower levels of all examined markers than PB-EPCs (Fig A). In co-culture with MSCs, PB-EPCs displayed the significantly higher levels of COL1, BMP4, VEGF, VEGF-R2, and THRMB than either VECs or BM-EPCs in co-culture with MSCs (Fig B). In a rabbit ulnar defect model, constructs cultured with MSCs and PB-EPCs displayed significantly enhanced regenerated bone mass after 12 weeks (Fig C, D). We have identified PB-EPCs, in combination with MSCs, to be a superior and clinically-relevant cell population for enhanced bone regeneration.
Characterisation of Mesenchymal Stem Cell Populations Used for Treatments of Rheumatoid Arthritis (RA) in a Murine Antigen-Induced Model

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A major challenge for translation of cell therapies to the clinic is the structural and clinical variability in clinical outcome, which can limit the ability to standardize treatment and provide the metrics required for regulatory approval. Use of animal models allows us to explore the relationship between cell quality and phenotype being used for treatment and the outcome in terms of reduction in inflammation of the joint which occurs in RA. Murine mesenchymal stem cells (mMSCs) were isolated from bone marrow of C57Bl/6 and Balb/c mice. Cells were tested for immunophenotype and their ability to form colonies and to differentiate into chondrocytes, osteocytes and adipocytes. 5 MSCs populations were subsequently used in antigen-induced arthritis model. One day after arthritis induction 500,000 mMSCs in serum-free medium were injected intra-articularly in the right knee joint of mice. Control animals were injected with serum-free medium. Knee joint diameter (swelling) was used as a standardised outcome measure and a clinical indication of joint inflammation. Our results demonstrated that level of joint inflammation was significantly reduced in MSC treated mice compared to control animals. MSCs from C57Bl/6 and Balb/c mice show different immunophenotype and differentiation potential. We were able to show a correlation in the level of inflammatory reduction and the ‘quality’ of the MSC population injected into the joint. Reduction in RA induced joint swelling over 7 days post MSC injection was determined by MSC quality parameters and indicators. In conclusion, the antigen-induced arthritis model is a relevant preclinical animal model which over a short time period allows assessment of input and output variables to be measured and assessed. This data can then be linked to manufacturing expertise to define cell parameters for quality markers.

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Inferior Ectopic Bone Formation of Mesenchymal Stroma Cells from Adipose Tissue compared to Bone-Marrow: Rescue by Chondrogenic Pre-Induction

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Human mesenchymal stroma cells derived from bone marrow (BMSC) and adipose tissue (ATSC) represent a useful cell source for bone tissue engineering. While ectopic bone formation is a standard activity for BMSC, this capacity for ATSC was so far unclear. Aim of this study was to judge the bone formation potency of ATSC in an ectopic mouse model and search for molecular differences between both cell sources. BMSC and ATSC (n=7) were cultured over 2 passages, seeded on β-TCP granules and implanted either directly or after 6 weeks of chondrogenic pre-induction into SCID mice. Bone formation of explants was quantified by histomorphometry. Molecular differences between BMSC and ATSC were assessed by RT-PCR. Although ATSC showed better proliferation than BMSC and displayed a similar osteogenic in vitro differentiation, they did not form bone within 8 weeks in vivo. Chondrogenic pre-induction of ATSC/β-TCP constructs installed bone formation in >75% of samples at comparable levels to BMSC. ATSC expressed less BMP-2, BMP-4, VEGF, angiopoietin and IL-6, and more adiponectin mRNA compared to BMSC. In conclusion, chondrogenic pre-induction was necessary to enable efficient ectopic bone formation by ATSC and to overcome their lower osteochondral commitment, reduced proangiogenic activity and altered immune modulation. This points towards a need for enhanced inductive conditions to make this more easily accessible cell source attractive for future applications in bone regeneration.
Crosstalk Between Human Progenitor Endothelial Cells And Mesenchymal Stem Cells Within A Three Dimensional Macroporous Polysaccharide-Based Scaffold

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Several studies have reported the benefit of mesenchymal stem cells for bone tissue engineering. However, vascularization remains one of the main hurdles that must be overcome to reconstruct large bone defects. In vitro prevascularization of three-dimensional (3D) constructs using co-cultures of human progenitors derived endothelial cells (PDECs) with human bone marrow mesenchymal stem cells (HBMSCs) has been described. The crosstalk between the two lineages has been studied in 2D but remains largely unknown in 3D. The aim of this study is to investigate the cell interactions between PDECs and HBMSCs in a porous matrix composed of polysaccharides, pullulan and dextran. This scaffold promotes cell interactions by inducing multicellular aggregates composed of a ring of PDECs around HBMSCs. Cell aggregation contributes to the formation of junctional proteins composed of Connexin43 (Cx43) and VE-cadherin and a resulting activation of osteoblastic differentiation of HBMSCs stimulated by the presence of PDECs. Inhibition of Cx43 by peptide mimicetic 43GAP27 induced a decrease of mRNA levels of Cx43 and of the bone specific markers. Finally, subcutaneous implantations in NOG mice revealed an increase of osteoid formation with the tissue-engineered constructs seeded with HBMSCs/PDECs compared with those loaded only with HBMSCs. In conclusion, this matrix offers a 3D microenvironment that favour cell communication, osteogenesis and bone formation.
Infiltrated Pancreatic Islet Derived Stem Cells in the Injured Rat Spinal Cord Reduces Lesion and Functionally Recovery

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Spinal cord injury is a very serious and common health problem, and it is still not curable functionally. Nowadays, stem cell based therapy is promising some valuable strategies for its functional recovery. Pancreatic islet (PI) derive from embryonic endoderm, but display features of neurons, including a shared set of cell-autonomous developmental regulators. Our group and others showed that nestin-positive progenitor/ stem cells isolated from human and murine pancreas. For this reason, we aimed to analyze the effect of rat PI-derived stem cells (rPI-SCs) delivery on lesion site after spinal cord injury, and to observe the functional recovery after transplantation. The experimental rat population was divided into three groups: laminectomy+trauma(1); laminectomy+trauma+PBS(2); laminectomy+trauma+SCs(3). Their motility were scored regularly. After 4-weeks, the tissue sections were analyzed for GFP labeled SCs and stained for vimentin, nestin, S100, BDNF, NF, GFAP, CNPase, vimentin and proinflammatory (MPO, IL-1β, IL-6,MIP-2) and anti-inflammatory (IL-1ra, prostaglandin E2 receptor) cytokines. The scores showed significant motor recovery in Group 3, especially in hind limb functions. GFP-labelled cells were localized on the injury site. Also decreased proinflammatory cytokines levels and increased the intensity of anti-inflammatory cytokines were determined. Thus, transplantation of rPI-SCs might be an effective strategy to improve functional recovery following spinal cord trauma.
Characterization of Endogenous Progenitor Cells Mobilized After Fracture Healing

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Endogenous cell homing, intended as an alternative method to attract cell populations already present in the body to injured sites represents a feasible and attractive option for in situ tissue regeneration. Indeed in response to injury, the interactions occurring between immune cells and platelet released factors create a suitable microenvironment, which brings about the recruitment of regenerative stem/progenitor cells, from either the blood or tissue-specific niches, toward the wound healing site. However the intrinsic characteristics of circulating progenitor cells and the precise mechanisms involved in the regenerative processes have been not fully elucidated. The aim of the project is to characterize and isolate specific subpopulations of endogenous progenitor cells mobilized after fracture healing. A transverse femoral shaft fracture was created in the right femur of 6 weeks old C57Bl/6 mice. The peripheral blood (PB) from mice underwent or not (control) a fracture was collected for flow cytometric analysis after 1, 3, and 7 days post-damage induction. We demonstrated that fracture induced the mobilization of a specific cell population (lineage$^-$ CD45$^-$ Ter119$^-$ 7-AAD$^-$) to the PB. Indeed, in the initial 3 days post-fracture, we observed a 2-fold increase of this cell population compared to control mice. The percentage of this specific population decreased after 7 days. These data may provide a novel insight into the mechanism of bone formation involving circulating progenitor cells, as well as lead to novel therapeutic strategies for improving repair of skeletal tissue.
Human Nasal Chondrocytes Can Efficiently form Bone in a Craniofacial Environment

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Fully differentiated human nasal chondrocytes (hNC) exhibit a phenotypical plasticity and a regenerative capacity for cartilage repair. While literature suggests that hNC are also capable of osteogenic differentiation, their bone forming capacity has never been demonstrated. Investigating if hNC could follow one or both of the archetypal routes of bone formation (endochondral and intramembranous), we engineered hNC-based grafts of hypertrophic induced cartilage or ceramic-based constructs in vitro, respectively, that were subsequently implanted subcutaneously into nude mice. Heterogeneity of the HNC whole population was characterized through a clonal study while environmental influences on bone formation were finally addressed by orthotopic implantation of constructs into a calvarian defect. NC-based tissues with characteristics typical of hypertrophy did not progress towards endochondral bone formation upon sub-cutaneous implantation. The minimal intramembranous bone formation observed (<5%) was in line with the relatively low (<30%) percentage of osteogenic hNC subpopulations derived by an independent clonal study. Following orthotopic implantation into a calvarian defect model (intramembranous bone repair), we were able to demonstrate that hNC were not only able to survive in a homotopic boney environment, but also able to actively contribute in new bone formation, contrary to a heterotopic cell source (mesenchymal stromal cells). Our findings show that homotopic environmental cues are essential for hNC to survive and actively produce intramembranous bone.
Development and Characterization of a New Ex vivo Model of Parkinson's Disease Using Organotypic Slices without Neurotoxin Injection

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Parkinson's disease (PD) is the second most frequent neurodegenerative disorder, characterized by the loss of the dopaminergic nigro-striatal neurons. Unfortunately, the current treatment induces undesirable side effects. Using brain organotypic slices, ex vivo models can be developed to mimic neurodegenerative disorders. These slices can be maintained in culture for many weeks and conserve the three-dimensional architecture of the brain and its microenvironment. They confer researchers a simple method to observe cellular interactions and mechanisms. We developed a new ex vivo model of PD using 400\textmu m organotypic slices of sagittal brain sections from rat neonates allowing observation of the whole nigro-striatal pathway. This easy, rapid and reproducible model mimics nigro-striatal degeneration observed in PD after a mechanical lesion, and does not imply dopaminergic neurotoxins. About 6 slices can be obtained per rat and can be conserved in culture during 16 days. Immunostaining against tyrosine hydroxylase (TH), the rate limiting enzyme in dopamine synthesis, allowed observing a loss of dopamine fibers in the striatum which reached 40\% in 3 days, while GABAergic neurons were not affected. This TH-positive fiber loss correlates with a diminution of dopamine striatal secretion quantified by mass spectrometry. We further studied the nigro-striatal pathway integrity by magnetic resonance imaging. A diminution of 60\% in 48h of the pathway integrity was detected, which may lead to the striatal dopaminergic denervation.

In conclusion we developed a new, rapid, reproducible and specific ex vivo model of PD based on a mechanical lesion allowing screening of novel therapeutic approaches.
Histopathological Analysis of Articular Cartilage Regeneration in Tissue Engineering Scaffolds

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The purpose of this research is to assess in vivo articular cartilage regeneration induced by tissue engineered scaffolds. These were macoporous poly(ethyl acrylate-co-hydroxyethyl acrylate) copolymer networks containing 90% of ethyl acrylate monomeric units, along with various cross-linking concentrations, obtaining 4 study groups with different stiffnesses. Pore structure was produced with template techniques. Biomaterials in form of discs were implanted in a 3mm chondral defect on adult rabbit knees, previously injuring subchondral bone to allow proper blood flow for cell repopulation purposes. Controls were submitted to similar layout, with no disc implantation. Animals were allowed to heal for 3 months. Regeneration was assessed through histological procedures. Implanted scaffolds induced articular cartilage regeneration on injured surface as well as cell colonization. An inverse association with the stiffness of the scaffold was observed, featuring increased fibrous tissue in harder scaffolds. Overall developing cell population within the scaffolds was immature, not well differentiated, forming cartilage and bone clusters, together with scarce blood vessels and multinuclei cells. Controls originated cartilage with fibrous appearance when compared to native hyaline cartilage. In conclusion, tissue engineered scaffolds induced cartilage regeneration on the injured articular surface, holding an inverse correlation with the stiffness of the biomaterial. Furthermore, cell colonization and integration with surrounding tissue was more fitting with softer biomaterials.
An In vivo Evaluation of Three-Dimensional Chitosan-Agarose-Gelatin Cryogel Scaffold for the Repair of Subchondrial Cartilage Defect in the Adult Rabbits

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In order to repair articular cartilage defect using tissue engineering approach chitosan–agarose- gelatin (CAG) cryogel scaffolds were used. These scaffolds provide three-dimensional support for the regeneration of the cartilage over the defect area. In this study, surgical method was used to create deep subchondral defect in the New zeland white rabbits. The CAG scaffolds were implanted over the cartilage defect area and the study was conducted for the time period of seven weeks. After two weeks macroscopic observation of the defect site revealed that the implanted scaffold was integrated with the surrounding tissue. Macroscopic observation of the scaffold implanted site also shows the process of healing of the defect area. Infiltration of the host cells in the cryogel was confirmed by histological staining and scanning electron microscopy of the implanted cryogel sections. Moreover, the regeneration of the cartilage was confirmed by the histological analysis of the treated joint; which showed that by the fourth/fifth weeks, the cartilage was regenerated. The hematological analysis indicates that implantation of CAG scaffold did not elicit any kind of adverse immunological reactions. ELISA was done to quantify the TNF-α level of the blood serum and it showed that its level was quite low and in the non toxic range throughout the experimental run. Animals with defects but no scaffold implantation were taken as control these animals were also studied for all the parameters which were analyzed for the scaffold treated animals. No sign of cartilage regeneration was observed in the control throughout the experiment period of seven weeks.
During guided bone regeneration (GBR), the bone defect is physically separated from soft tissue to facilitate bone regeneration. The cellular and molecular determinants of the regeneration process, and the influence of the membrane properties on this process are not described. This study aimed to evaluate the bone regeneration under novel cholesterol-bearing pullulan nanogel and collagen membranes, and to relate that to the in vivo cellular and molecular activities during GBR. Defects of 2.3 mm diameter were created in rat tibiae. The defects were covered with nanogel membrane, collagen membrane or left as sham. After 6 and 21d, histological, radiographical and immunohistochemical analyses were performed. Further, the gene expression in the GBR membrane-adherent cells and in bone formed under in the defects was analysed using qPCR. Radiographical (micro-CT) analysis revealed higher bone mineral density in the defects covered with the nanogel. Histological observations showed a thicker cortical layer under the nanogel. Immunoreactivity for osteogenic marker periostin was stronger in nanogel-covered defect. Higher expression of osteogenic growth factors (BMP-2, and TGF-beta) was detected in cells adherent to nanogel membrane and in bone formed in the defect under this membrane. The expression of osteogenic differentiation genes (ALP and OC) was higher in cells adherent to the collagen membrane. No difference was observed for the latter genes in the bone formed in the defects of the 3 experimental groups. It is concluded that by changing the properties of the membranes it is possible to alter the cellular and molecular activities during GBR, resulting, subsequently, in improved quantity and quality of bone formed under the membranes.
Multichamber Three-dimensional Systems Enable in vitro and in vivo Screening of the Immunocompatibility of Biomaterials

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The present study aimed to analyze the tissue reaction of three different collagen materials within a multichamber three-dimensional (3D) system, which we already showed to be useful for discovering regenerative medicine therapies. This system allows the screening of different experimental conditions in vivo while reducing the animal number with comparable results to single material implantation. Rapid prototyping was used to generate a 3D 3x3 multichamber (1.5x1.5 mm) made by 300PEOT55PBT45. Each device row was filled with one collagen material. Plain devices served as controls. In a parallel study, the same collagen membranes incubated with human monocytes were implanted into SCID-mice. Histological analysis and established histomorphometrical methodologies were applied after early and late points: (10 to 60 days). In mice the devices were intact without any visible material break down. Three different cellular tissue reactions to the materials were distinguished differing in their in vivo vascularization. In vitro cell-based experiments were performed within the device comparable to those in standard well-plates. Human monocytes were involved in material degradation and enhanced the implantation bed vascularization. The 3D multichamber system delivered comparable results as observed with the same materials used as plain scaffolds. This system allows for in vitro and in vivo investigations, and for characterization of material-specific inflammatory responses. This innovation could serve as a platform to help keep necessary in vivo experiments to an ethically acceptable minimum.
Arteriosclerosis remains a main cause of death. Stenting and vessel replacement by native or synthetic grafts are well-established techniques in clinical practice. Unfortunately, the patency rates remain unsatisfactory. We are investigating a tissue engineered stent graft with the aim of a minimally invasive treatment. The proposed concept consists of a self-expanding Nitinol stent covered by a fibrin gel lined with autologous endothelial cells. The BioStent grafts were successfully implanted as interposed carotid artery grafts in sheep for up to 13 weeks. Myofibroblasts and endothelial cells (ECs) were harvested from sheep carotid artery. The stents were embedded in fibrin gels (5mg/ml) including autologous smooth muscle cells/myofibroblasts, seeded with ECs and conditioned dynamically for 3 weeks under physiological pressure. The conditioned stents were crimped, released and implanted into the carotid of the sheep up to 13 weeks. The grafts were observed in vivo by sonography and angiography and by scanning electron microscopy (SEM) and histological staining after explantation. All implanted vessels remained patent during the observation time. No thrombosis, hyperplasia or discontinuity at the anastomosis was observed. SEM revealed a complete endothelial cell lining of the luminal surface of the stent without lesions. The tissue engineered BioStent graft performed well in the arterial circulation of sheep, withstanding the pressure conditions and remaining patent. The obtained stents underwent a crimping-release process prior implantation as a first evaluation of the robustness of the endothelial layer in vision of a minimally invasive delivery.
A thermoresponsive Poly-caprolactone Scaffold for 3D In vitro Stem Cell Differentiation and In vivo Angiogenesis

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In tissue engineering (TE) strategies, the natural process of regeneration is imitated by using bioresorbable scaffolds that support cellular attachment, migration, proliferation and differentiation. Based on the idea of combining a fully degradable polymer (Poly(ε-caprolactone) with a thermoresponsive polymer (polyethylene glycol methacrylate) a scaffold was developed [1], which liquefies at 4°C and solidifies at 37°C. In this study, it was combined with either C2C12 cells or human adipose derived stem cells (ASCs) to generate an expandable 3D construct for soft or bone TE. As a first step, biomaterial seeding was optimized for C2C12 cells as well as ASCs and their attachment, survival, distribution and persistence within the 3D material was characterized. C2C12 cells were differentiated towards the osteogenic as well as myogenic lineage, while ASCs were differentiated with adipogenic or osteogenic media. Differentiation was examined using qRT-PCR for the expression of osteogenic, myogenic and adipogenic markers. C2C12 cells differentiated towards the myogenic lineage and ASCs treated with adipogenic differentiation medium showed increased expression of specific markers in 3D compared to 2D, suggesting that the thermoresponsive scaffold qualifies for 3D in vitro differentiation towards soft tissue. Next, we evaluated whether the scaffold was able to support vascularization in an in vivo angiogenesis model. An increase in newly formed vessels was detected during the first two weeks followed by a decline in total vascularization in week four. No signs of inflammation were visible. This suggests that the scaffold supports angiogenesis and is therefore a promising candidate for further TE approaches.

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An Investigation of the Ability of IL-10 Overexpressing MSCs to Delay or Prevent Osteoarthritis Progression in Mice

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Osteoarthritis (OA) is a disabling degenerative joint disease affecting synovial joints, which is characterised by loss or damage of articular cartilage. Synovial inflammation is believed to contribute to both symptoms and disease progression. The use of adult mesenchymal stem cells (MSCs) to modulate/prevent disease progression is an area of active investigation. In recent years there has been a paradigm shift in the mode of action of MSCs from direct tissue formation to modulation of the local environment by release of anabolic and immunomodulatory factors. Given the role of inflammation in OA it is logical that anti-inflammatory factors such as interleukin 10 (IL-10) might also delay or prevent OA progression. The aim of this study was to investigate the ability of adult MSCs overexpressing IL-10 to delay or prevent OA in a collagenase induced model. 1U of collagenase (in 6ml) was injected into the knee joints of C57BL/6 mice twice over 2 days. One week later animals were treated with intra-articular injection of 20,000 adult human MSCs (in 6ml saline) or MSCs overexpressing adenoviral Epstein Barr Virus IL-10. Adnull expressing MSCs, vehicle and adIL-10 alone were used as controls. After 7 weeks legs were harvested for histology and draining lymph nodes were taken to assess the effects of MSCs and IL-10 on T and B cell populations. Following treatment there was a decrease in the presence of CD4 and CD8 positive T cells in the popliteal lymph nodes in IL-10 transduced MSC treated animals compared to other groups. Scoring of the degree of arthritis is underway with regard to effects on serum cytokine production and OA progression as determined by the OARSI scoring of whole joint histological sections.
In vitro Chondrogenesis and In Vivo Repair of Osteochondral Defect with Human Induced Pluripotent Stem Cells

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The purpose of this study was to test the in vitro chondrogenic potential and in vivo capacity for cartilage regeneration of human iPS (hiPS) cells. We used the hiPSC line (SBI, cat# SC802A) generated by direct delivery of four proteins fused to a cell penetrating peptide. After 21 days of in vitro culture, pellets were analyzed for DNA contents, GAG amount, and the expression of chondrogenic markers. The hiPS-pellets or alginate-hiPSCs constructs were implanted in the osteochondral defect model rat. Mesodermal markers increased in EB while undifferentiated ES markers disappeared. After 21 days of chondrogenic culture in micromass pellets, GAG analysis showed that proteoglycan production was significantly greater in chondrogenic pellets than in undifferentiated hiPSCs and EBs. Safranin-O staining demonstrated that the cells in chondrogenic pellets took on the appearance of immature chondrocytes and secreted extracellular matrix. The chondrogenic marker gene and protein expression increased after 21 days of pellet culture. The chondrogenic pellets derived from hiPS cells have very low expression of hypertrophic or osteogenic markers. Also, hiPS cells underwent good chondrogenic differentiation in PLGA scaffold or alginate gel as well. When hiPS cells in either pellet state or in alginate hydrogel were implanted in the osteochondral defects created on the patellar groove of immunosuppressed rats, the defects implanted with chondro-induced hiPS cells showed a significantly better quality of cartilage repair than the control defects. In conclusion, this study provides a proof-of-principle strategy for using hiPSCs as a cell source for cartilage tissue engineering.
Peripheral arterial disease (PAD) is one of the major vascular complications in the elderly and is associated with significant morbidity and mortality. The gold-standard treatments for severe PAD are surgery or endovascular revascularization. Adult stem cell therapy associated with three-dimensional, scaffolds represents a promising strategy for vascular regeneration. In addition, it has been shown that scaffolds architecture and structure plays an important role to dictate stem cell behavior. Electrospun gelatin B scaffolds with random or aligned fibers were prepared. Human bone marrow derived MIAMI cells were loaded onto the scaffolds and subsequently implanted in an aged mouse model of critical limb ischemia (femoral artery ligation and excision) to determine the angiogenic potential of our cellular constructs. Following two weeks of implantation, the vessel beds of mice treated with aligned scaffolds yielded long straight vessels parallel to the nanofiber alignment in the scaffold. In contrast, fewer vessels with more disorganized orientation were formed when mice were treated with MIAMI cells seeded on scaffolds with random fiber orientation. Importantly, aligned fiber orientation induced superior levels of leg reperfusion in comparison to random fibers. Electrospun gelatin B scaffolds with variable fiber orientations developed by dynamically controlling the electrostatic field during the electrospinning process have the ability to direct cellular behavior and morphology. We demonstrated that cellular constructs provide directional cues to newly formed vessels and lead to improve functional recovery of the ischemic region.
Osteoarthritis (OA) is characterized by the progressive destruction of articular cartilage, subchondral bone remodeling, and synovial deterioration, reducing the quality of life. In this study, the efficacy of an intra-articular injection of Adipose derived Stromal Cells (ASC) in an OA rabbit model was explored with MicroCT (Bruker MicroCT 1172) for a 3D evaluation of changes in femoral bone. Eight weeks after OA induction by bilateral Anterior Cruciate Ligament Transection (ACLT), 2x10^6 and 6x10^6 autologous ASC, isolated from rabbit inguinal fat, were injected into the affected knees; control group was injected by 4% of Rabbit Serum Albumin (RSA). At 16 and 24 weeks, Epiphyseal Bone volume Fraction (BVF), Trabecular Thickness (TbTh), Trabecular Separation (TbSp), Subchondral cortical Bone Thickness (SBT) and osteophytes volume (V) were evaluated. Positive effects of ASC administration on cartilage tissue were detected in a previous experiment (Desando G, et al. Arthritis Res Ther. 2013. 29;15(1):R22[Epub ahead of print]). At 24 weeks a slight increase in differences between values (in BVF, TbTh, TbSp) of medial and lateral condyle was observed in the treated groups. The highest SBT values were found in the medial condyle at 24 weeks where the load is greater than in other knee areas. In the experimental groups there were no significant differences in V values in medial condyles over time, despite an increase in lateral ones (Fig.1). These preliminary MicroCT data revealed a positive contribution of ASC in contrasting degenerative events occurring during OA, with a little remodeling of subchondral bone. A longer follow-up is probably necessary to observe changes in bone.

Fig. 1 – Microtomographic sections. 3D model of rabbit condyle treated with ASC cells compared to control group (4% RSA – Rabbit Serum Albumin)
Engineering a Functional Hematopoietic Microenvironment Through Human Mesenchymal Stromal Cells-Based Endochondral Ossification

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The aim of this study was to establish a hMSC-based model of ectopic ossicle capable to support functional hematopoiesis through endochondral ossification. Expanded hMSC were seeded onto 8 mm diameter, 2 mm thick collagen sponges (Ultrafoam\textsuperscript{TM}, Davol Inc.), cultured for 5 weeks in vitro under chondrogenic and hypertrophic conditions and then implanted subcutaneously in nude mice. Consistently with the fact that bone regeneration requires an inflammatory environment we added IL-1β to the hypertrophic medium and assessed its effect on in vitro mineralization, hypertrophy, extracellular matrix processing and in vivo remodelling and bone formation. Samples were analyzed by histology, IHC, Luminex\textsuperscript{®} assays, ISH for human Alu repeats and µCT. Bone marrow cells, extracted after 12 weeks from the implanted samples were analyzed by flow cytometry and transplanted into lethally irradiated congenic animals. In vivo, extensive remodeling with stabilized vessel ingrowth (CD31\(^+\); NG2\(^+\); αSMA\(^+\)) and osteoclast activity (TRAP\(^+\), MMP9\(^+\)) took place. In particular, IL-1β resulted in (i) enhanced MMP13 endogenous activity; (ii) enhanced osteoclasts activity by increased M-CSF levels and RANKL/OPG ratio; (iii) faster vascularization; (iv) larger regions of bone marrow, possibly because of an increased synthesis of SDF1, IL-8, M-CSF and MCP-1. Murine bone marrow cells in the newly generated bone included phenotypically and functionally defined HSC at a comparable frequency than normal bones of the same mice. This work reinforces the evidence of self-organization ability of hMSC-based hypertrophic cartilage templates into functional HSC niches and prompt for further fundamental investigations on the biology of bone development and of HSC niches.
Programmed Cell Death and Local Defence Reactions around the Biomaterials Covered by Mesenchymal Stem Cells and Fibrin Emulsion

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Biocompatibility research covers the combination between biomaterial and different factors stimulating implantation. Mesenchymal stem cells (MSC) and blood factors are supposed to give positive biostimulation. The aim of work was the detection of apoptosis and local immune response of soft tissue after different factors and hydroxyapatite/tricalciumphosphate (Hap/TCP) implantation. Experimental tissues were obtained from 5 rabbit spine subcutis 5 months after Hap/TCP implants. The experimental side enrolled by MSC covered biomaterial and Hap/TCP with fibrin emulsion implanted near the biomaterial in 3.5 months. Control consisted of the same rabbit other body side where only biomaterial was implanted. Routine staining, TUNEL and IMH for IL10 and defensin 2 were used. Results demonstrated inflammation, connective tissue capsule, neoangiogenesis in fibrin and Hap/TCP side. Defensin-containing cells were detected in various numbers of control and Hap/TCP covered by MSC side while after biomaterial and fibrin few to moderate cells showed defensin. IL10 positive structures showed similar numbers in all tissue. Apoptosis affected control with following decrease in biomaterial and fibrin side and notable decrease in Hap/TCP covered by MSC side. MSC covered biomaterial decreases apoptosis still 5 months after implantation while fibrin emulsion shows similar not so distinct effect. Fibrin emulsion near the biomaterial raises massive inflammation in tissue. However, similar IL10 expression after different factors and biomaterial combinations suggests about equal anti-inflammatory response of tissue. The variations in defensin 2 expressions prove the individual tissue response.
Bioartificial urothelium offers an innovative therapeutic option for urethral reconstructive surgery especially for the surgical treatment of long and complicated urethral strictures. In this study a Collagen Cell Carrier (CCC) seeded with autologous urothelial cells was used for urethroplasty in minipigs after induction of a urethral stricture. Six minipigs underwent urethral stricture induction by thermocoagulation. Porcine urothelial cells (PUC) were isolated from bladder tissue, expanded, labelled by PKH26, and seeded onto the CCC in high density. After seven days of stratification minipigs were treated with autologous urothelium-CCC-transplants for urethroplasty at stricture side. Animals were sacrificed after one, two, and four weeks and urethral tissues were examined histologically and via immunofluorescence staining of AE1/AE3, CK20, p63, E-cadherin, and ZO-1. The seeded cell carrier displayed excellent stability characteristics when applied in minipigs. Transplanted cells could be detected via positive PKH26 fluorescence in all animals. Urothelium-matrix transplants integrated well into the host tissue without any severe inflammation. Throughout the complete experimental period none of the animals showed symptoms of rejection or stricture recurrence. Immunofluorescence analysis confirmed epithelial phenotype, junction formation as well as ongoing differentiation two weeks post-op.

Autologous urothelial cells seeded on CCC were suitable for urethroplasty in minipigs. This study suggests cell-seeded CCC for urologic reconstruction purposes and recommends its use with regard to clinical application. Funding: The study was supported by Viscofan BioEngineering Germany.
A Feasibility Study of New Tubular Constructs for Urinary Diversion in a Porcine Model

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Uretero-ileo-cutaneostomy according to Bricker has been the primary choice of surgeons for urinary diversion. However, the use of gastrointestinal (GI) tissue for this procedure is associated with severe complications. Regenerative medicine may provide a solution by developing alternatives for GI tissue. In this study, three differently reinforced tubular scaffolds (l=12 cm, Ø=15 mm) were prepared from bovine collagen type I and (i) a slow-biodegradable Vypro polymer (Ethicon), (ii) a fast-biodegradable Vicryl polymer (Ethicon) or (iii) an additional layer of compressed collagen, to maintain an open structure. After freeze-drying, cross-linking and sterilization, three different constructs were closed at one end and extra-peritoneally attached to bladders of 11 female Landrace pigs. Pigs were evaluated by loopogram at 1 or 3 months post-implantation and sacrificed for microscopic evaluation by histology. Successful implantation was achieved in all pigs, with a survival rate of 80% (1 related and 1 unrelated death). Loopograms showed normal bladder capacity in all pigs. Handling and suturing was easiest for the Vicryl construct. After 1 month, both the Vicryl and dual layered constructs were resorbed. The Vypro construct was still present after 3 months, resulting in stone formation in 66% of the animals. The end result of the Vicryl and dual layered construct was superior, indicated by urothelial cell coverage, neovascularisation and muscle ingrowth after 3 months. The best regeneration was observed in the Vicryl constructs. This study shows the applicability of a new collagen-based tubular scaffolds, reinforced with a fast-degradable and clinical applicable Vicryl polymer mesh as a potential construct for urinary diversion.
The Role of Macrophages During the Foreign Body Reaction

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Macrophages are a pivotal cell type during the foreign body reaction (FBR). They orchestrate the pro-inflammatory microenvironment inside and around biomaterials by secretion of mediators such as chemokines, cytokines and growth factors. Outside the biomaterial these factors may instruct the fibroblasts that produce a fibrous capsule around the biomaterial. Inside the biomaterial these factors attract and activate more inflammatory cells. Additionally, macrophages mediate the degradation of biomaterials through secretion of matrix-degrading proteinases and phagocytosis. In this study we investigated what happens during the FBR when macrophages are not present. Hexamethylene diisocyanate-crosslinked collagen scaffolds were implanted in “Macrophage Fas-Induced Apoptosis” (MaFIA) mice, which allow induction of macrophage depletion. We observed that macrophage depletion completely inhibited ingrowth into the scaffolds and resulted in an increased capsule size. QPCR analysis revealed decreased expression levels of pro-inflammatory mediators such as TNFα and IL1β, and increased expression levels of collagens and fibroblast-stimulating growth factors such as EGF, FGF1, FGF2 and TGFα. Our results indicate that macrophages are indeed crucial for the generation of a pro-inflammatory microenvironment inside implanted biomaterials, leading to inflammatory ingrowth. In contrast, macrophages do not appear to be important for the generation of a fibrous capsule around implanted biomaterials. In fact, our data suggest that the macrophages present in the capsule might instruct the surrounding fibroblasts to produce less fibroblast-stimulating factors and less collagens.
The purpose of this study was to investigate the regenerative efficacy of an injectable and biocompatible hyaluronic acid/mildly crosslinked alginate hydrogel (HA/ALG hydrogel) containing human adipose-derived mesenchymal stem cells (hAdMSCs) for vocal fold (VF) regeneration. The HA/ALG hydrogel was designed as a cell carrier to contain hAdMSCs. The hAdMSCs encapsulated within HA/ALG hydrogel were injected into the VFs of rabbits immediately after direct injury. Endoscopic evaluations were performed at 1 and 3 months after injury. Functional evaluations of mucosal vibration and viscoelastic properties were carried out post-euthanization at 3 months after injury. Histopathologic and immunohistochemical evaluations of extracellular matrix (ECM) components in VFs were conducted. Engraftment and in vivo differentiation of implanted hAdMSCs were investigated by detecting fluorescent-labeled cells. The administration of hAdMSCs or hAdMSCs in HA/ALG hydrogel resulted in better macroscopic morphologies and viscoelastic properties than the administration of PBS. In particular, hAdMSCs encapsulated within HA/ALG hydrogel-treated VFs showed less collagen deposition than PBS-injected VFs. Immunohistochemistry revealed that the amount of collagen type I in hAdMSCs encapsulated within HA/ALG hydrogel-treated VFs was less than PBS-injected VFs. hAdMSCs in HA/ALG remained viable in recipient VFs at 1 month after transplantation and some of them were observed to be differentiated into VF fibroblasts. The findings of the present study suggest hAdMSCs encapsulated within HA/ALG should be viewed as a potential therapeutic candidate for VF tissue regeneration and HA/ALG hydrogel is a promising biomaterial for prolonging the retention time of stem cells in VFs and fostering fibroblastic differentiation.
Endothelial Progenitor Cells Promote Neovascularization of Tissue Engineered Implants

In vivo

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The sufficient supply with nutrients and growth factors is of critical value for tissue engineered implants. In large size implants the ingrowth of vessels often fails. Thus, neovascularization has to be promoted by the implant. The use of co-cultures of mesenchymal stem cells (MSC) and endothelial progenitor cells (EPC) are a promising therapeutic approach for tissue engineered implants with the ability to integrate in the surrounding tissue. The aim of the current study was to promote neovascularization (in vitro, in vivo) of hydroxyapatite-containing polyurethane (PU) scaffolds. MSC and EPC were isolated from human bone marrow using Ficoll and MACS\textsuperscript{®} respectively. PU scaffolds were seeded with MSC and EPC in different proportions in the presence of autologous platelet lysate. Constructs for in vitro observation were incubated in a medium suitable for both osteogenic and endothelial differentiation. For in vivo analysis constructs were implanted subcutaneously in nude mice and excised after 8 weeks. Neovascularization was examined on cryosections stained with toluidine blue or in immunofluorescence staining of endothelial marker. We observed neovascularization in the scaffolds in vitro and in vivo. Most importantly, in vivo a perfusion of scaffolds was observed, which was never detected in cell-free scaffolds, confirming anastomosis of new formed vessels in the scaffold with the host vascular system. In conclusion, we could demonstrate that the presence of EPC is highly effective to induce neovascularization in tissue engineered constructs.
Bone Defects Repair in Rats Activated by Scaffolds of Biosilicate Associated or not with Low Level Laser Therapy.

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Between the resources studied in order to accelerate the bone repair process is the LLLT. To stimulate the osteoblast proliferation we can see many bioactive materials like bioglass, and recently many papers shows a novel bioactive material (Biosilicate) to fill bone defects. This study aimed to assess the effects of bioactive material (Biosilicate) associated or not with LLLT irradiation on consolidation of induced tibial bone defects in the rats. A total of forty male Wistar rats (± 300 g) were randomly divided into four groups, with 10 animals each: group control bone defect without any treatment (GC); group bone defect irradiated with LLLT (GL); group bone defect treated with implantation of Biosilicate scaffolds (GB); group bone defect treated with implantation of Biosilicate scaffolds and irradiated with LLLT (GBL). The animals were submitted to laser irradiation (830 nm, continuous wave, 100 mW, 0.028 cm\textsuperscript{2}, 3.57 W/cm\textsuperscript{2}, at 120 J/cm\textsuperscript{2}, energy of 3.4 J, Ti=34 s) at a single point on the bone defect for eight sessions, on alternate days. Morphological analysis revealed that the laser treatment group showed better tissue organization in relation to other groups. Furthermore, morphometric analysis revealed that the only irradiated animals showed a higher amount of newly formed bone if compared to the group using only Biosilicate (p≤0.05). The association of treatments was not different from controls. From the results obtained in this study, it is possible to suggest that the LLLT treatment had higher osteogenic potential at time point 15 days after surgery. The bone repair process was faster and well organized in the group using LLLT treatment when compared to the use of biomaterial alone or even when the two treatment modalities were associated.

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Bioresorption Characteristics of Plain and Cell-loaded Polyhydroxyalkanoate and Polycaprolactone Film Scaffolds in Chronic Long-term Experiment


Federal State Budgetary Institution Research Institute for Complex Issues of Cardiovascular Diseases under the Siberian Branch of the Russian Academy of Medical Sciences, Russia, Kemerovo

The study was aimed at examining the biodegradation speed of polyhydroxyalkanoate and polycaprolactone copolymer compositions, potentially suitable for small vascular graft production, with and without multipotent mesenchymal stromal cells derived from the bone marrow. Light microscopy was used to assess the bioresorption characteristics of film scaffolds made out of polyhydroxybutirate/valerate (PHBV) and polycaprolactone (PCL) after subcutaneous implantation to rats. Inflammatory infiltration of the surrounding tissues in the implantation site was found to be moderate or focal lymphohysteocytic infiltration and to stay not more than 1 month. The surface fragmentation of 7.5% PHBV and 10% PCL scaffolds occurred after 8 months; multichamber thin-wall capsules were formed with no signs of perifocal inflammation. 5%PHBV and 10% PCL scaffolds did not have any obvious signs of destruction after 12 months. The presence of multipotent mesenchymal stromal cells derived from the bone marrow on the surface delayed the start of copolymer scaffold bioresorption for 1 month. Thus, the optimal co-polymer composition for a vascular graft scaffold is 5%PHBV and 10% PCL. The presence of MMSC does not reduce the biodegradation time of copolymer scaffolds.
An adipose-derived mesenchymal stem cell (ASC)-seeded acellular dermal matrix (ADM) has been used for bone regeneration. Additionally, numerous studies have demonstrated that low-level laser therapy (LLLT) affects bone repair in experimental animals. This study investigates the feasibility of using an ASC-seeded ADM along with LLLT to repair bone defect in athymic nude mice. Critical-sized calvarial defects were treated either with an ADM (ADM group), an ADM along with LLLT (ADM/LLLT group), an ASC-seeded ADM (ADM/ASCs group) or an ASC-seeded ADM along with LLLT (ADM/ASCs/LLLT group). The animals were euthanized at 3, 7, 14, 21, 28 and 56 days. Micro-computed tomographic (CT) imaging, histological evaluation, immunohistochemistry and western blot were performed. In micro-CT images, the ADM/ASCs and the ADM/ASCs/LLLT groups showed remarkable bone formation after 14 days. Furthermore, bone regeneration in the ADM/ASCs/LLLT group was obvious at 28 days, but in the ADM/ASCs group at 56 days. Bone mineral density (BMD) and bone tissue volume (BTV) in the ADM/ASCs/LLLT group significantly increased after 7 days, but in the ADM/ASCs group after 14 days. Histological analysis revealed that the defects were repaired in the ADM/ASCs and the ADM/ASCs/LLLT group, while the defects in the ADM and the ADM/LLLT groups exhibited few bone islands with fibrous connection at 28 and 56 days. The successful seeding of ASCs onto ADM was confirmed, and LLLT enhanced the proliferation and the survival of ASCs at 14 days.

An ASC-seeded graft promoted bone regeneration, and the application of LLLT on an ASC-seeded ADM results in rapid bone formation. The implantation of an ASC-seeded ADM combined with LLLT may be used effectively for bone regeneration.
Efficient Repair of Cartilage Defect Using Chondrocyte/silk Based Scaffold Constructs

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Today's repair of articular cartilage defects using suitable scaffolds that have optimal structural and mechanical properties, excellent biocompatibility, controlled degradation rate, and good handling characteristics has remained a continuing challenge. In the present work, a highly porous silk-based scaffold was developed from \textit{Bombyx mori} cocoons by freeze-drying method and fully characterized via morphology, mechanical properties and biodegradability. The isolated chondrocytes from human hyaline cartilage loaded on scaffolds and cultured in a spinner flask bioreactor. Chondrocyte/silk constructs were subsequently implanted subcutaneously into nude mice for 12 weeks in order to determine whether the cartilaginous tissue structures could be formed \textit{in vivo}. Histological staining showed the cell/scaffold implants formed mature and well-developed cartilaginous tissues that were similar to the native cartilage, as evidenced by chondrocytes within lacunae. In a rabbit osteochondral defect model, H & E and Alcin blue staining reveals that the groups which were treated with chondrocytes/scaffolds, exhibited cartilaginous tissue while the groups leaved by scaffold alone, formed fibrous tissues. Moreover, integration of cell/scaffold constructs into the surrounding native cartilage was much better than scaffold alone. Indeed, the engineered constructs showed accumulation of extracellular matrix including type II collagen and glycosaminoglycan. These results are quite promising for the successful repair of human articular cartilage defects using chondrocytes/silk based scaffold constructs in the future.
Formulation and Evaluation of Microencapsulated Hydrogen Peroxide for Controlled Release of Oxygen for Improvement of Tissue Regeneration In vitro and In vivo

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The lack of oxygen has been an obstacle in the regeneration of large volume engineered tissues. To overcome this limitation, our group developed an oxygen generating microspheres via direct encapsulation of H2O2 into PLGA using a double emulsion solvent evaporation technique to provide physiologically relevant concentrations of oxygen for adequate tissue regeneration over a three dimensional scaffold. These H2O2-containing microspheres were subsequently encapsulated in alginate based hydrogels where catalase was immobilized. In this system, H2O2 decomposes to oxygen and water without production of free radicals by catalase. We investigated this oxygen generating material in vitro and in vivo. Animal studies with BALB/c nude mice include H&E staining, immuno-histochemistry and western-blotting and imaging. It was observed that encapsulated H2O2 in the hydrogel can produce an efficient amount of oxygen at a controlled release manner to enhance skeletal muscle regeneration. BLI imaging of transplanted cells also revealed that oxygen significantly enhance the cell population upto 14 days as compare to no oxygen sample. Moreover, up regulation of myosin, myogenin, and CD31 expression was found in oxygen supplied group. Thus oxygen generating biomaterial complex can play an important role in cell based tissue regeneration facing oxygen diffusion limitation in tissue engineering.
Articular Cartilage Repair by Transplantation of Human Umbilical Cord Blood-Derived Mesenchymal Stem Cells in a Rabbit Model

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The purpose of this study was to assess the feasibility of human umbilical cord blood derived mesenchymal stem cells (hUCB-MSCs) transplantation for the repair of articular cartilage defects in a rabbit model. Full-thickness articular cartilage defects of 3-mm diameter were created in the femoral trochlear grooves of both knees in 24 rabbits. Then, the animals were divided into two groups: group A, no treatment at control knee and implantation of hyaluronate gel at experimental knee; group B, implantation of hyaluronate gel at control knee and transplantation of composite of hUCB-MSCs and hyaluronate gel at experimental knee. At 16 weeks post-treatment, repair of articular cartilage defect was evaluated grossly, histologically, immunohistochemically and semiquantitatively. Implantation of hUCB-MSCs with hyaluronate resulted in overall superior cartilage repair tissue with better quality than implantation of hyaluronate alone or no treatment. The cellular architecture and collagen arrangements were similar to those of adjacent normal articular cartilage tissue, and the repair tissue contained a considerable amount of hyaline cartilage. Human umbilical cord blood-derived mesenchymal stem cells in combination with hyaluronate gel seems to be useful for the repair of full thickness articular cartilage defects.
Bioactivated Surgical Sutures Promote Wound Healing

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Delayed wound healing and scar formation are among the most frequent complications after surgical interventions. Although biodegradable surgical sutures present an excellent drug delivery opportunity, their primary function is fixation. Mesenchymal stromal cells (MSCs) act as trophic mediators and are successful in activating biomaterials. Here biodegradable sutures were filled with adipose-derived MSCs to provide a pro-regenerative environment at the injured site. Results showed that after filling, MSCs attach to the suture material, distribute equally throughout the filaments and remain viable in the suture. Among a broad panel of cytokines, filled sutures constantly release vascular endothelial growth factor and stromal derived factor-1α to supernatants. Such conditioned media was evaluated in an in vitro wound healing assay and showed a significant decrease in the open wound area compared to controls. After suturing in an ex vivo wound model cells remained in the suture and maintained their metabolic activity. Furthermore, cell-filled sutures provide the ability to be cryopreserved. This study presents an innovative approach to equip surgical sutures with pro-regenerative features and allows the treatment and fixation of surgical wounds in one step. Therefore MSC-filled sutures represent a promising tool to promote wound healing after injury.
Epithelial Cell-Cell Junctions Development and Maturation in a Heterotypical Skin and Oral Mucosa Model Generated with Human MSC

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New cell sources allowing the generation of human bioengineered skin and oral mucosa without the need to obtain autologous biopsies is required. In this work, we developed 3D models of human skin and oral mucosa using stromal cells in combination with biomaterials and human umbilical cord Wharton’s jelly stem cells (HWJSC) to determine the differentiation capabilities of these cells to oral mucosa and skin keratinocytes at in vitro and in vivo levels. Three-dimensional heterotypical models of artificial oral mucosa (H-hOM) and skin (H-hS) were generated using fibrin-agarose biomaterials and primary HWJSC, oral mucosa and skin fibroblasts cell cultures, and epithelial cell-cell junctions were evaluated by plakoglobin (PKG) immunohistochemistry and transmission electron microscopy analysis (TEM) after in vivo implantation in athymic nude mice. Results and discussion: Our results showed that H-hOM and H-hS implanted in athymic mice were gradually positive for PKG, with slight and irregular expression at days 10 and 20 and intense homogeneous expression on the cell surface from the 30th day of in vivo implantation. TEM analysis confirmed the presence of well-formed epithelial cell-cell junctions. Conclusions: These results suggest that HWJSC have potentiality to differentiate to oral mucosa and skin epithelial cells in vivo and could be an appropriate novel cell source for the development of human oral mucosa and skin in tissue engineering protocols.

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Guided Bone Regeneration By Chemotaxis-induced
Mesenchymal Stem Cell Recruitment

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Guided bone regeneration (GBR) is a frequently used strategy to treat bony defects in craniomaxillofacial region, involving surgical placement of a membrane to create and maintain a secluded space. To date, most of commercial GBR membranes show dominant capability as barrier membranes for space provision, however, an unsolved problem remains that the membranes lack biological activity to stimulate the in situ bone defect regeneration. In the past decades, many attempts have been made to incorporate growth factors into conventional GBR membranes to enhance their biological activity. Recently, an alternative approach has emerged, which focuses on cell recruitment. This cell recruitment approach maximizes the body’s own regenerative capacity by recruiting endogenous stem/progenitor cells to the injury site to exert local functional and reparative effects, and to synchronize the biological functions of other cell types via paracrine mechanisms. The goal of this work was to develop a bioactive membrane with cell recruiting capacity. To this end, GBR membranes were prepared by electrospinning using poly(ε-caprolactone) (PCL) blended with type B-gelatin, and functionalized with stromal cell derived factor-1α (SDF-1α) via physical adsorption. The results showed that the local release of SDF-1α from PCL/gelatin membranes significantly increased in vitro bone marrow stromal cell recruitment and yielded enhanced bone formation in rat cranial defects, indicating a potential usage of such membrane for GBR strategy.
Mesenchymal stromal cells (MSCs) are a promising cell source for tissue engineering therapies aimed at forming new articular cartilage or endochondral bone. Controlling the chondrogenesis of MSCs to become either permanent articular cartilage or hypertrophic cartilage destined to be replaced by endochondral bone has remained challenging. During natural limb development both tissues are formed from the same cartilaginous anlage. Remarkably, permanent articular cartilage resides in a hypoxic environment while that of hypertrophic cartilage is normoxic. We therefore hypothesized that the oxygen tension is a key signaling event that steers the chondrogenesis of MSCs into either permanent articular or hypertrophic cartilage. Human bone marrow derived MSCs that were chondrogenically differentiated under hypoxia (2.5% O₂) produced more hyaline cartilage and potent inhibitors of hypertrophic differentiation e.g. GREM1, FRZB and DKK1. In contrast, exposure to normoxia (21% O₂) inhibited the expression of these markers and coincided with the formation of hypertrophic cartilage. Strikingly, hypoxic or normoxic precultured MSCs behaved quite distinctively when subcutaneously implanted in nude mice. Where hypoxia preconditioned implants remained cartilaginous, normoxia preconditioned implants readily underwent calcification, vascular invasion and endochondral ossification. In short, this study reveals that oxygen tension is a key factor steering the chondrogenic differentiation program of MSCs in either permanent cartilage or hypertrophic cartilage. Modulating the oxygen tension provides a simple yet effective mechanism for future tissue engineering therapies to control the chondrogenic fate of MSCs.
In vivo Evaluation of a Bioengineered Model of Artificial Maxillary Bone


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Cleft palate is the most common serious congenital anomaly that affects the orofacial region and multiple surgical techniques have been used to repair these deformities. Staphylorraphy was initially described by Graefe in 1819 and consists of repairing the cleft palate defect by using the adjacent oral mucosa tissues. This procedure results in uncovered sites of oral mucosa that may affect the normal development and growth of the bone palate. In this work we have analyzed the effect of grafting a bioengineered oral mucosa construct on palate regeneration in a staphylorraphy model. Newborn New Zealand rabbits were randomly assigned to 4 study groups. In each group, a palatal defect was surgically generated and the gap was unrepaired (control group) or repaired with free Biobrane graft; acellular fibrin-agarose grafts; and cellular fibrin-agarose grafts with Biobrane grafts. Quantitative histological analyses of the bone area and grafted oral mucosa were carried out on postoperative day 120 using H&E and picrosirius staining. Animals with a complete oral mucosa covering, showed adequate bone development and orthotypical bone patterns, whereas unrepaired animals had midline palatal deviations and reduces bone diameter. These results demonstrate that the normal growth and development of the palate are highly influenced by the bone coverage by oral mucosa and suggests that bone should be covered with oral mucosa during staphylorraphy.

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PL-co-DLA Based Scaffolds and Mesenchymal Stem Cells in Meniscus Regeneration

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The advent of tissue engineering on bioresorbable polymer substrates and subsequent implantation can improve the healing process in meniscal injuries. The aim of this study was to evaluate the ability of mesenchymal stem cells seeded on polymer scaffolds to stimulate regeneration of the whole meniscus.

Porous poly(L-co-D,L-lactic acid)/polycaprolactone-triol (PL-co-DLA/PCL-T) and poly(L-co-D,L-lactic acid-co-trimethylene carbonate) (PL-co-DLA-co-TMC) scaffolds were obtained by solvent casting and particulate leaching. New Zealand rabbits underwent total medial meniscectomy. Both knees in each rabbit were used after which implants consisting of unseeded scaffolds or seeded scaffolds were introduced into the medial meniscus; the control group consisted of rabbits that received no implant. Twenty four weeks after implantation gross evaluation showed the presence of well-formed neo-meniscus maintaining the original anatomical structure on both scaffolds. The histological results revealed the generation of fibrocartilaginous-like tissue demonstrating the construct’s biocompatibility. Furthermore, mature fibrocartilaginous tissue mimicking native meniscal tissue structure was achieved using seeded PL-co-DLA-co-TMC scaffold, presenting aligned collagen bundles. Thus, this study showed the efficiency of using polymer scaffolds in fibrocartilage regeneration after meniscectomy and the full potential of mesenchymal stem cells tissue engineering lacking chemical growth factors.
Tissue degeneration and impaired regeneration are considered major contributors to enhanced tendon injury and trauma with increasing age. Epidemiological studies indicate an association of the rate of tendon ruptures and age, presenting a major challenge in an ageing society. However, the molecular and cellular mechanisms underlying this phenomenon remain poorly defined. To identify candidate genes contributing to the degenerative, age-related process in tendons we performed a suppression subtractive hybridization screen comparing cDNA libraries generated from pooled RNAs extracted from Achilles tendons of mature-adult (3 months; n=10) and old (18 months; n=9) female C57BL/6 mice. Differential screening identified a total of 90 potentially differentially expressed candidate genes in mature versus old mouse tendons. The majority of the identified cDNAs encoded for extracellular matrix (ECM) family members, including decorin, biglycan, fibromodulin, fibronectin, thrombospondin-1, as well as collagen type-1 and type-3. In addition, the ECM proteases MMP-2 and MMP-9 were identified. Last but not least, lysyl oxidase was found to be differentially expressed in young and old Achilles tendons. The expression levels of these genes were confirmed by quantitative RT-PCR, showing all of these transcripts to be down-regulated in old tendons. Next to the aforementioned candidate genes, the expression level of the tendon-progenitor associated marker protein Scleraxis was also decreased in old mouse tendons. The molecular mechanisms underlying the diminished transcription of these genes and their potential contribution to an increased risk of tendon degeneration and injury with age are under current investigation.
In vivo Investigations of the Early Stages of Bone Healing with Microdialysis

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The therapy of critical-size bone defects represents a significant clinical problem. To improve bone healing, it is important to characterise the active molecules and cellular mechanisms in the early stages after the injury. Therefore the authors developed microdialysis in bone defects to identify mediators and growth factors in the early stages of bone healing. A 5 mm defect was created in the femur of male Wistar rats and stabilized with an internal fixation system. The microdialysis catheter was placed within the defect, fixed to the skin and the dialysate was collected continuously under anaesthesia for 24 hours. The dialysates were analysed by ELISA, HPLC/MS and Proteome Profiler™ array. Determination of protein concentration showed that it was possible to collect protein continuously from the bone defect with the chosen experimental setting. The total protein concentration varied between 0.15 mg/ml and 0.44 mg/ml. We were able to determine Interleukin-6 (IL-6) and transforming growth factor-β1 (TGF-β1) concentration over 24 hours. IL-6 was secreted within the first 3 h and the highest concentration was measured between 12 and 15 h after the injury. The analysis of the samples by HPLC/MS illustrated that the more than 1000 collected proteins can be assigned to biological processes like immune response, cell-cell-signalling, blood coagulation, inflammatory response and others. By Proteome Profiler™ array further interesting chemokines like CXCL1 and CXCL7 could be detected in the dialysate which should be analysed in detail.

In conclusion microdialysis appears to be a valuable method to characterise molecular changes during the early stages of bone healing.
Effect of Adipose Mesenchymal Stem Cells and Plasma Rich in Growth Factors in the Treatment of Osteoarthritis Disease in Dogs

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Osteoarthritis is one of the most challenging medical problems. Regenerative therapies promote regeneration instead of tissue repair, inducting cellular agents. Both adipose mesenchymal stem cells (aMSC) as the PRGF have proved their effectiveness in several medical disciplines. The aim of the study was to evaluate the use of aMSC and PRGF in the treatment of OA in dogs. 34 dogs with elbow, knee or hip OA, were randomly assigned to one of the following groups:

- **PRGF:** 2cc of autologous PRGF (Anitua’s method) (n=29).
- **aMSC:** intraarticular injection 2cc with 30 millions of autologous aMSC (Stem Cells method) (n=23). Dogs were evaluated in basal, 1, 3 and 6 months after treatment with Bioarth Osteoarthritis Scale, owner’s questionnaire and VAS scale. The nonparametric Kruskal-Wallis and Mann- Whitney tests were used to compare non-categorical variables and crosstabs with contingency coefficient was used to evaluate the categorical ones.

OA degree was assessed radiologically did not vary within groups. However, in functional limitation, joint movement, flexion and extension degree, range of movement, owner’s and veterinarians perception of pain (VAS) and owner satisfaction of the treatment, a clear improvement has been seen since the first month of study, maintaining up to six months. However, the aMSC group obtained better results at 6 months than the PRGF group in joint movement (p=0.014), flexion degree (p=0.015), extension degree (p=0.01), veterinarians VAS (p=0.00) and in owner’s satisfaction with the treatment. There were no adverse effects present during the study.

Also PRGF and aMSC improve the functionality and quality of life in all degrees of OA.
Collagen-Coated Biodegradable Polymer Scaffolds for Bone Tissue Engineering Application

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Collagen coating of biodegradable polymer scaffolds have been widely performed in Tissue Engineering to improve the biocompatibility of scaffolds by the mechanism of biorecognition. This study aimed at evaluating the modified surfaces of poly (l-co-D, lactic acid)-PLDLA scaffolds, by acrylic acid grafting (PLDLA-AAc) followed by type I collagen immobilization (PLDLA-Col). The surface characterization revealed that collagen was deposited on scaffold surface as a stable layer, with a net-like fibrillar collagen structure in non-specific areas of the PLDLA-Col scaffolds. ATR-IR spectra of coated scaffolds exhibited absorption peaks at 1662 and 1559 cm⁻¹, typical of amide I and II. Scaffolds cytocompatibility was evaluated in vitro by osteoblastic-like cell culture. The result have shown that PLDLA-Col scaffolds significantly improved adhesion and cell proliferation rates, as well as stimulates collagen synthesis (p<0.01). Biocompatibility of PLDLA-Col scaffolds was assessed by using a critical-size bone defect with 8mm, created in Wistar rat calvarial bone. Sixty rats were used and divided in three treatments: Control (the critical-size defect was kept empty), unseeded PLDLA-Col and PLDLA-Col scaffolds seeded with osteoblast-like cells for 10 days. After 8 and 12 weeks of implant. Histological and histomorphometry results have shown that both PLDLA-Col and seeded PLDLA-Col scaffold improved the progression of bone healing, however, seeded PLDLA-Col promoted greater (p<0.01) new bone formation in both peripheral and central area of the defect, suggesting that collagen coating method is a potential strategy for the development of biofunctional biomaterials for tissue engineering application.
Therapeutic Approach to Peripheral Arterial Disease Using Transfer Printing of Cell Layers from Bioactive Hydrogels

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Cell-based therapy is an attractive approach for repair of damaged ischemic tissue. In the present study, we developed cell-interactive peptide (RGD) incorporated thermosensitive hydrogels for harvesting muscle tissue-like construct and investigated their effect on therapeutic angiogenesis. We cultured C2C12 myoblasts on the hydrogels for 24 hr to form a tissue-like layer and then transferred it to hindlimb ischemia model generated in mice. These processes based on tissue-like constructs cultured on thermosensitive hydrogels can be simply transferred on native tissue by tissue transfer printing technique due to size change in response to the temperature stimuli. We found that the transferred monolayer of C2C12 myoblasts at transplantation region was retained in a longer period of time than injected cell suspension without any diffusion. The anti-fibrotic effect on the C2C12 myoblast layer was confirmed by histochemistry of the ischemic hind limb tissue. In addition, Laser Doppler Perfusion data (28 days) also supported that C2C12 cell layer had better therapeutic effects on the ischemic tissue. Consequently, our system would represent a new approach for therapeutic angiogenesis.

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Selection of Viable Adipose-Derived Stem Cells for the Generation of a Functional 3D Bone Model for Cleft Palate Defects

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Generation of artificial tissues is highly dependent on the use of cells with high viability potential. The use of viable stem cells is a requirement for the elaboration of a functional artificial bone to repair bone defects. Cleft palate is a relatively common congenital malformation in which a significant part of the palate bone is lost. Availability of an artificial viable bone tissue could contribute to improve the results obtained to the date. In this work we have generated a bioengineered bone using selected viable stem cells for use in cleft palate defects. Primary cultures of rabbit adipose-derived stem cells (ADSC) were established using collagenase II. Evaluation of the cell viability was carried out by trypan blue exclusion analysis and live/dead\textsuperscript{®} assays of cells corresponding to 8 consecutive cell passages. Then, the most viable cells were used to generate a fibrin-agarose nanostructured bone substitute which was kept in culture for 35 days and it was grafted in vivo in a rabbit model of cleft palate. Histological analysis was carried out by H&E, alizarin red and osteocalcin detection in control and differentiated samples. Our results showed that the bone substitutes showing the most orthotypical bone patterns corresponded to the constructs generated with the most viable cells (cell passages 6 and 7). Alizarin red staining and osteocalcin detection showed positive pericellular signal after 28 days of in vitro culture. These bone constructs were useful for the repair of cleft palate defects in the analyzed rabbit model, suggesting that ADSC could have clinical usefulness.

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Transplanted Umbilical Cord Mesenchymal Stem Cells Modify the In-vivo Microenvironment Enhancing Angiogenesis and Leading to Tissue Regeneration

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The umbilical cord represents a widely available source of mesenchymal stem cells (UC-MSC) which could be promising candidates for tissue regeneration therapies. Current literature on the osteogenic capacity of UC-MSC is still controversial. Hence, the goal of the present work was to clarify the role of UC-MSC in bone regeneration. We used clinical-grade UC-MSC isolated from Wharton’s Jelly and cultured with human Platelet Lysate (hPL). A cytokine array assay of UC-MSC conditioned medium revealed the presence of numerous cytokines such as IL-8, IL-6, MCP-1, PAI-1, CXCL1, G-CSF and MIF indicating a pro-inflammatory role. It also revealed high concentrations of VEGF as detected by ELISA. This pro-angiogenic feature was confirmed using a metatarsal angiogenesis assay. Since it has now been established that a pro-inflammatory initial burst is important for neoangiogenesis and consequently bone regeneration, we hypothesized that this characteristic of UC-MSC secretome could influence the behavior of the cells in vivo. UC-MSC were seeded on ceramic scaffolds and implanted subcutaneously in immunocompromised mice. Histological analysis showed that UC-MSC were not able to form bone tissue but numerous blood vessels were noted. Interestingly, live-cell tracking in vivo showed that UC-MSC remained viable and proliferative for only 12 days after implantation. However, preliminary flow cytometric analysis of retrieved cells demonstrated the involvement of UC-MSC in the recruitment of host putative osteoprogenitor cells. We speculate that an orthotopic rather than ectopic site of implantation could create the proper environment to support UC-MSC mediated angiogenesis and recruitment of host osteoprogenitors in the lesion.
Use of Rat Adipose-Derived Mesenchymal Stem Cell to Accelerate the Neovascularisation of Interpolation Flaps in Rats

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Interpolation flaps are widely used in Plastic surgery for reconstruction of large or deep defects. However waiting 2–3 weeks for the pedicle division is still a challenging problem that increases the cost of treatment and the duration of hospital stay. In order to solve this problem, based on angiogenic potential of stem cells accelerating neovascularization and improving flap survival of interpolation flaps, our aim was to divide the pedicle earlier.

We studied in two main groups and four subgroups of rats. Each animal underwent creation of identical cranially based skin flaps. Under each flap of the experimental group green fluorescent protein (GFP) labeled adipose derived stem cells was injected into the flap’s distal portion; into the recipient bed and into the wound margins and medium solution in the control group in the same fashion. At a postoperative interval of 5, 8, 11 and 14 days, each proximal pedicle of four rats from each group was divided. At 7 days after the operation, flap survival was measured. According to statistical evaluation, stem cell-treated group showed a significant increase in flap viability compared to the control group. Immunofluorescence staining revealed that all endothelium samples in experimental group were GFP (+) (all of those in the control group were negative).

It seems that rAD-MSC treatment markedly increases experimental interpolation flap survival and may be of clinical importance to divide the pedicle earlier.
Compartment syndrome (CS) is a serious complication arising from a variety of extremity injuries and resultant swelling within the fascicles of the muscle tissue. The developments of new techniques to treat CS include angiogenic therapy, anti-fibrosis treatments and stem cell therapy, all which aim to enhance tissue regeneration and functional recovery. We have developed a rat model of CS that represents an ideal system for testing cellular, biological and pharmacological agents for the restoration of skeletal muscle volume and function. Neonatal blood pressure cuffs were placed on the hind limbs of nude rats. A pressure of 120-140 mm Hg was held for 3 hours to induce compartment syndrome. Muscle progenitor cells (MPCs) derived from GFP+ mice were injected into the tibialis anterior muscle 4, 7 and 11 days after injury. Muscles were collected at 14 and 28 days after injury for histological analyses. Peak tetanic isometric force, as determined via neural stimulation, was used as the measure of muscle function. Our rat injury model is a viable model for the testing of cell therapies for CS. We were able to demonstrate that GFP+ MPCs were able to survive, integrate and differentiate into mature innervated myofibers when injected into CS injured tissue.

MPC therapy may prove efficacious for the treatment of skeletal muscle injuries. This model of injury is a viable means of testing further cell and biological therapies for the treatment of CS.
Changes of FoxP3 Expression by Hypoxia Regulate the Homing and the Therapeutic Effects of Placenta-derived Mesenchymal Stem Cells in a Rat with CCl4-injured Liver

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Mesenchymal stem cells have potentials including differentiation, self-renewal, and immunomodulation. Recently, placenta-derived mesenchymal stem cells (PD-MSCs) have been introduced as an alternative cell sources considering their strong immunomodulatory effect. Forkhead box P3 (FoxP3) known as a master of regulator T involves migration of MSCs. However, the efficacy of PD-MSCs through the alteration of FoxP3 and homing-related factors by hypoxia are unclear. Here, the objectives were to analyze the expressions of FoxP3, integrins, and Rho family in CCl4-injured tissues engrailed with PD-MSCs and compare their relationships. In addition, the efficiency of PD-MSCs to liver tissues of CCl4-injured rat through intrasplenic transplantation (STx) and intravenous transplantation via the tail vein (TTx) were evaluated. PD-MSCs invasion was increased in hypoxia. Expressions of HIF-1α in rat liver were more increased in both STx and TTx than non-transplantion (NTx) (p<0.05). Increased HIF-1α induced to alter the expressions of integrins, pFAK and pRac/cdc42, and FoxP3 in tissues. Integrins were increased in tissues after PD-MSCs transplantation. Especially, the expressions of integrin a4, HIF-1α and pRAC/cdc42 in the spleen were significantly increased than liver (p<0.05). Interestingly, there are shown dynamic alteration of FoxP3 mRNA expression in liver tissues depends on transplantation routes and decreased FoxP3 expression in liver tissues by intrasplenic transplantation of PD-MSCs trigger to hepatic regeneration in CCl4-injured liver rat model. Taken together, enhanced homing activity of PD-MSCs by hypoxia could be control the efficacy of liver regeneration through the alteration of FoxP3 and integrin signaling.
Standardization of Isolation and Culture Protocols of Human Adipose-derived Stem Cells (hASCs) using Xeno-free Products and In vivo Assessment of the Potential of these Cells combined with Starch-Polycaprolactone Scaffolds for Bone Regeneration

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The advantages of using human adipose-derived stem cells (hASCs) in tissue engineering and regenerative medicine are well recognized. Nevertheless, the transplantation of these cells when exposed to animal-derived reagents presents severe risks, and for this reason, it is essential to standardize isolation and culturing procedures, and also to assess the potential of ASCs in pre-clinical models, documenting their safety and efficiency in specific regenerative strategies. Therefore, the main aims of this study were to explore these technical challenges testing alternative non-animal sources of collagenase and trypsin-like enzymes for the isolation and passage of ASCs¹⁴ and assessing (using a rat critical cranial defect model) the regenerative potential of these cells combined with a scaffold material (starch-polycaprolactone –SPCL fiber meshes) in a bone TE strategy. Results obtained demonstrated that it is possible to use purified enzymes without decreasing the yield of stromal/stem cells nor affecting their surface markers⁵ and differentiation potential. The outcomes from the in vivo assays shown osseointegration in defects filled with in SPCL loaded with hASC engrafted calvarial defects as compared to control groups (non-loaded scaffolds and empty defects) that showed little healing. In summary, it was demonstrated that non differentiated human ASCs enhance ossification of critical nude mice calvarial defects, and SPCL proved to be a suitable biomaterial for bone tissue engineering.

These outcomes have practical implications in the development of Standard Operating Procedures for cGMP of clinical grade human ASCs, which are essential for their future use in the clinical practice.
Conjoined Therapeutic Effect of Udenafil and ADSC/BDNF-Membrane System in a Rat Model of Cavernous Nerve Injury

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To prevent cavernous nerve injury and corpus cavernosum apoptosis-induced erectile dysfunction after prostatectomy surgery, we investigated whether oral administration of Udenafil combination with covering adipose-derived stem cells (ADSCs) and brain derived neurotrophic factor (BDNF) immobilized PLGA membrane on the injured cavernous nerve could further improve erectile dysfunction. Adult SD rats were divided into five groups: normal group (sham-operated group), BCNI group (bilateral cavernous nerve crush injury group), Udenafil group (oral administration of Udenafil 20 mg/kg daily), AB group (BCNI group with ADSCs covered with BDNF membrane on cavernous nerve), AB/Udenafil group (AB group with Udenafil group). After four weeks, erectile function was examined prior to tissue harvest. Penile tissues were evaluated in terms of the expression of smooth muscl actin (SMA), neuronal nitric oxide synthase (nNOS), and vascular endothelial growth factor (VEGF). Cyclic guanosine monophosphate (cGMP) level of the corpus cavernosum was quantified by cGMP assay. AB/Udenafil treatment markedly improved erectile function and prevented the architecture damage of the corpus cavernosum, compared with other treated groups. Udenafil had no statistical significance on increasing nNOS expression, but enhancing VEGF expression. On the contrary, AB group had no statistical significance on enhancing VEGF expression, but increasing nNOS expression. AB/Udenafil treatment significantly increased nNOS expression, VEGF expression, and elevated cGMP level, compared with Udenafil group and AB group. The orally administered Udenafil combination with ADSC/BDNF-membrane system protected cavernous nerve and improved angiogenesis in the corpus cavernosum, which further maintained erectile function in a rat model of post-prostatectomy erectile dysfunction.

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Chronic kidney disease affects 5-7% of people in the world and the shortage of organs has driven a strong interest in engineering new kidney tissue de novo, from single cells. Starting from suspensions of embryonic kidney cells, we recently constructed “organoids” that can carry out kidney functions when implanted into a living animal. Here, we built on this technology to generate a human-mouse chimeric organoid suitable for transplantation. Dissociated renal cells from E11.5 mouse embryos were centrifuged in the presence of labeled human umbilical cord-mesenchymal stem cells (UC-MSCs), and the pellet was cultured in vitro. Immunofluorescence analysis revealed only minimal integration of UC-MSCs into developing renal structures, with the majority of the cells to be clustered in extra-tubular areas. In order to enhance the integration and differentiation of human stem cells in the self-forming tissue, UC-MSCs were transfected with GDNF, a key factor in kidney organogenesis. GDNF-expressing cells integrated efficiently into the renal tissues, which developed into homogenous, fine-grained chimeric organoids. At 1 day, GDNF-transfected cells were organized into NCAM+ induced metanephric mesenchyme, and expressed the renal specific marker Pax-2. At 5 days, chimeric nephrons elongated, and were bounded by laminin+ basement membranes. Implantation of chimeric organoids in vivo under the kidney capsule of an athymic rat host resulted in further growth and maturation of these tissues as assessed by histochemical analysis.

These results provide the initial proof-of-concept for a technology to engineer kidney tissue from in vitro expanded human stem cells, an ability which can potentially solve the problem of finding sources of tissues or organs.
Low Intensity Ultrasound Has a Therapeutic Potential for Lowering Brain Edema Formation in Rats

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Brain edema is a major contributing factor to morbidity and mortality of a wide variety of nervous system disorders. In brain edema, there is a net increase in the water content of the brain parenchyma which results in an increase in the brain tissue volume and as a consequence, intracranial pressure is increased which causes brain herniation, irreversible brain damage, and ultimately, death. In this study, we examined whether low intensity ultrasound (LIUS) stimulation can reduce brain edema formation. Cytotoxic edema was induced in the rat hippocampal slices in vitro by incubation in artificial cerebrospinal fluid (ACSF) solution in oxygen glucose deprivation (OGD) condition. We found that OGD conditions induced edema, and LIUS stimulation attenuated edema in the hippocampal slices. Further, our in vivo study showed that LIUS stimulation significantly reduced the whole brain water content and intracranial pressure in rats. As water channel protein Aquaporin 4 (AQP4) has been implicated in brain edema formation, we asked whether the lowering of edema formation by LIUS stimulation is related with AQP4. We found that the membrane localization of AQP4 in the astrocytic foot processes was increased in the edematous hippocampal slices, but was decreased in the LIUS-stimulated slices. Furthermore, we found that AQP4 expression was markedly decreased in the microvessels of the cerebral cortex and hippocampus in the LIUS-stimulated rats. Our data suggested that LIUS stimulation attenuated cytotoxic brain edema formation possibly through a decrease in AQP4 activation and lesser membrane localization of AQP4.
Preclinical evaluation of bone regeneration activities in segmental sheep tibia defects were treated with single or triple dose of injectable tissue-engineered bone. Female sheep aged between 1-3 years old and weighed between 20 to 25kg were used for the study. Critical size defect of 3cm was created at the mid diaphyseal region of the left tibia and care was taken to ensure the entire periosteum surrounding the defect was removed. To obtain mechanical stability, an external fixator (using IMEX Veterinary Inc SK™ system) was inserted in the routine manner. Injectable bone consisted of differentiated autologous mesenchymal stem cells, plasma and ceramic powder was prepared. Three weeks after the creation of the segmental tibia defect and stabilization using external fixators, one group was administered with single dose injectable tissue engineered bone and another group was treated with three doses of injectable tissue engineered bone. Sheep with untreated defects acted as controls. All animals were monitored radiographically. At the end of week 9 post implantation, animals were euthanized and the right tibias were obtained. Histological and CT-scanning (Skyscan 1076) were performed on the experimental tibias. X-ray view revealed little or no regeneration in both control and experimental sheep. No bone union occurred and only little bone regeneration found in both control and injected sheep. Micro-CT quantification showed significant more new bone was regenerated in injected groups. However, no difference in the amount of bone regeneration between single and triple doses of injectable bone. No additional benefits were found in multiple doses of injectable bone.
Recently, cell-based therapies, regenerative medicine, and tissue engineering have been progressing rapidly. We have developed a novel strategy for regenerative medicine to recover tissue functions using temperature-responsive cell culture surfaces. To overcome conventional methods such as the usage of single-cell suspension injection, we have applied transplantable cell sheets fabricated with temperature-responsive culture surfaces for cell delivery. In the field of gastroenterology, these regenerative medicine and tissue engineering approaches have attempted to prevent postoperative stricture by structurally and functionally reconstructing normal tissues through the promotion of early re-epithelialization after endoscopic large size mucosal resection. Our group previously reported a method of regenerative therapy involving the transplantation of fabricated autologous oral mucosal epithelial cell sheets in a canine model and demonstrated its human clinical application. So far, the endoscopic technique of cell sheet transplantation was not easily procedure, and there were no endoscopic delivery devices to be useful for cell sheets transplantation. Presently, we are developing two types of novel endoscopic device for cell sheets transplantation, and we also show recent our research for esophageal regeneration using cell sheet engineering after circumferential endoscopic large size mucosal resection. We examined allogeneic epidermal cell sheet transplantation using a novel endoscopic delivery device in order to transplant more than one cell sheet at the same time in porcine. The novel devices were designed with a computer-aided design system, and the three-dimensional data were transferred to a 3D printer. The surface of the cell sheet transplantation device was fabricated using FDA-sanctioned acrylic material. And then, primary epidermal cells were isolated from the lower abdominal skin of miniature pigs, cultured for 18 days at 37°C on temperature-responsive culture inserts. Transplantable cell sheets were harvested from the inserts by reducing temperature to 20°C. Immediately after creating full circumferential esophageal endoscopic submucosal dissection (ESD), an allogeneic epidermal cell sheet was endoscopically transplanted to the ulcer site. The pigs were endoscopically monitored, and sacrificed 2 weeks after transplantation. 2-3 pieces of the epidermal cell sheets (20mm in diameter) were successfully transplanted onto the ulcer site after circumferential ESD. In addition, early epithelialization and moderate stricture were observed by a number of transplanted cell sheets. These endoscopic delivery devices for cell sheet would enable easily transplantation of cell sheets onto the lumen of the esophagus. Additionally, fabricated allogeneic epidermal cell sheets might be useful for prevention of stricture after esophageal ESD as well as autologous oral mucosa epithelial cell sheets in swine model.
Biological Effect of Gellan Gum on Postoperative Intraperitoneal Adhesion Formation in the Rat Model

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An antiadhesion membrane made from gellan gum was fabricated and characterized. A 12-μm thick membrane of gellan gum was prepared and reacted with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide to obtain a cross-linked membrane (G/A70) with 87% gel content and a tensile strength of 46.5 MPa. In vivo, the G/A70 membrane had 90% less tissue adhesion. Inflammation-related and extracellular matrix protein gene expression in a rat model of abdominal surgery was found by real-time quantitative reverse transcription polymerase chain reaction analysis. On day 3, after surgery, the gene expression of ceruloplasmin and type V collagen in the G/A70-treated group was 1.9 and 0.3 times that of the control group, respectively. The G/A70 membrane elicited mild inflammation but suppressed type V collagen synthesis and reduced the occurrence of tissue adhesion. These findings may provide insights into the properties of gellan gum antiadhesion membranes and help to overcome problems involving tissue adhesions in surgical procedures.
Innovative clinical approaches to repair damage to bone tissues are being developed, including low-level laser therapy (LLLT). Particularly, LLLT is a common modality used to treat many skeletal muscle conditions and it seems to have an osteogenic potential. Although in vitro and in vivo data on the irradiation of bone defects by LLLT are encouraging, there is a clear need to understand the cellular and molecular modifications induced by this therapy which controls bone formation. In this context, the present study aimed to evaluate the temporal modifications of LLLT on the healing process of tibial bone defects in rats. Sixty male Wistar rats with bilateral tibial defects were randomly distributed into two experimental groups: control group (CG) and laser irradiated group (LG). Laser irradiation (830nm, 100 J/cm², 30mW) started immediately after surgery and it was performed for 1, 3 and 7 sessions. Animals were euthanized at 12 hours, 3 and 7 days after surgery. The qualitative histological analysis showed that the LG presented minor blood clots, intense inflammatory infiltrate around the defect when compared with CG at 12 hours after surgery. At 3 days, moderate inflammatory infiltrate and highly cellularized granulation tissue could be observed only in the LG. At 7 days, the CG presented inflammatory infiltrate, granulation tissue and some deposition of primary bone. In the LG minor inflammatory infiltrate, intense granulation tissue highly cellularized and newly formed bone were observed. The immunohistochemistry analysis showed that LG produced a higher expression of RUNX-2 at 12 hours after surgery when compared to CG. On 3 and 7 days, the immunoeexpression of RUNX-2 could be observed mainly in the control group. Our findings indicate that laser therapy improved bone healing process, by accelerating the deposition and organization of newly formed bone and activating osteogenic factors as RUNX-2 on created bone defects in tibias of rats.

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The purpose of this study is to evaluate the new bone density in rat skull defect using the new Chitin-Hydroxyapatite-Fibroin membrane by means of microscopic computerized tomography (micro CT) analysis. In this experiment, using Fifty-four rats, the membrane was applied on bony defect of 8mm on skull. The experimental group(n=18), Chitin-Hydroxyapatite-Fibroin membrane was used. In positive group(n=18), an absorbable collagen membrane(Bio-Gide®) was used. In negative control group(n=18), membrane was not used. We euthanized six animals by CO₂ at 2,4,8 weeks post surgery in each groups and then the volume & density of bone were measured and analyzed. Comparing groups of using or not using membrane, there were significance differences in volume & density of regenerative bone. Comparision between experimental and positive group, although the bone volume level of positive group was higher than experimental group, the difference was not significant. Bone density measurements have similar patterns with the results of bone volume. At postoperative 4 weeks we found significant difference between the Bio-Gide® group and experimental group. At 8 weeks after operation, there was no significant difference among all groups in bone density.

When using membrane for reconstruction of bone defect, Chitin-Hydroxyapatite-Fibroin membrane has similar bone regeneration ability compared with collagen membrane(Bio-Gide®).
Bone Regeneration Potential of the New Chitosan Based Alloplastic Biomaterial

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Alloplastic bone substitute materials are raising some more interest as an alternative for autologic transplants and xenogenic materials especially in oral surgery over the last few years. These not immunogenic and completely resorbable biomaterials became the basis for complete and predictable guided bone regeneration in many cases. The objective of our research was to evaluate the dynamics of bone formation in rats’ skulls after implantation of the new chitosan / tricalcium phosphate / alginate biomaterial in comparison to the commercially available alloplastic bone graft. 45 adult male rats weighing 300-400g were used for the study. The 85mm-diameter defects in calvaria bone were prepared with a trephine bur, and then filled with the bone substitute materials: chitosan / tricalcium phosphate / alginate (CH) or easy-graft Classic, Degradable Solutions AG (EA) or left just with the blood clot (BC). Animals were sacrificed at 1 and 3 months for histological, histomorphometrical and micro tomography evaluations. Histological evaluation at 1 month showed early new bone formation observed around the experimental biomaterial (CH). There were no features of purulent inflammation and necrosis, or granulomatous inflammation. Microscopic examination after 3 months from the surgery revealed trabecular bone formation around chitosan based bone graft with no significant inflammatory response. Different and less satisfactory results were observed for the commercially available EA and control BC.

The tested material (CH) showed a high degree of biocompatibility and osteoconductivity in comparison with the control groups and seemed to be a “user friendly” material for oral surgeons.
Use of Adipose-derived Mesenchymal Stem Cell to Improve the Viability of Composite Grafts: Experimental Study


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Today, especially condrocutaneous composite grafts are among the reconstruction options for tumor resection and posttraumatic nasal alar defects. The biggest problem with composite grafts is their limited viable area. Thirty six adult Wistar rats were divided into six groups. In Groups 1, 2, 3: Grafts were placed immediately after defect was created. In Groups 4, 5, 6: Grafts were placed 4 days after defect was created. In groups 1 and 4, rAD-MSC was injected; in groups 2 and 5, medium solution was injected. In groups 3 and 6, no injection was made.16 days after graft placement, scintigraphic agent was injected; then, leaving around a 1 cm intact area graft was excised and all rats were photographed. The survival area of each graft was measured by millimetric analysis. After scintigraphic images taken, all sections taken from grafts were examined histologically. According to statistical evaluation, stem cell-treated group showed a significant increase in viable area of composite graft compared to the control and medium groups. The ratio of the scintigraphic activity obtained by composite grafts at stem cell group were found to be significantly higher than the control group. Histologically, the capillary density in the composite graft tissue was clearly increased in stem cell-treated group, when compared with the control group. However in the experiment, we used GFP to label the adipose-derived stem cells. Immunofloresans staining revealed that all composite graft samples in stem cell group had lower levels of apoptosis and were all GFP (+). In this study; concluded that rADMSC treatment markedly increases experimental condrocutaneous composite graft survival and may be of clinical importance for treating skin and cartilage containing defects.
Hyaluronic Acid Tetrasaccharide Labeled with Tritium and Study of its Biodistribution in Rats

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Hyaluronic acid (HA) is a natural glycosaminoglycan composed of repetitive disaccharide units, each consisting of N-acetylglucosamine and D-glucuronic acid. This polysaccharide is an important part of the extra-cellular matrix, vitreous humour and skin. In our previous works we studied biodistribution of higher molecular weight HA modified with various types of radioactive markers. This presentation describes a biodistribution of the radioactively labeled HA tetrasaccharide, which is the smallest product of the standard enzymatic degradation. The synthesis is based on the reduction of the aldehyde-oxidized HA with $[^{3}H]$ sodium borohydride and subsequent enzymatic degradation with a hyaluronidase. The isolation was performed using gradient liquid chromatography using Luna NH₂ column and sodium phosphate as a mobile phase. Results showed that method is simple, fast, inexpensive, and may yield products with high efficiency, high specific activity and high stability. Unlike known chemical procedures based on oxidation-reduction strategy, this methodology produces labelled HA oligomers in the native form. It was observed that the liver is the major site of circulating HA tetramer uptake and degradation. The HA fragments are further released into the blood and then eliminated by the kidney. The stability of labeling was proved by the analysis of the released urine after 2 and 24 hours, where the presence of the radiolabeled HA tetramer was clearly detected.
Migrating Cells in Niche Regions And Cartilage of The Intervertebral Disc and in the Knee Joint Observed In vitro and In vivo. An Experimental Study In The New Zealand White Rabbit

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Potential stem cell niches(SN) was recently reported in intervertebral discs(IVDs) and knee joints(KJ) in different mammals. The aim here was to examine possible migration of cells from SN involved in IVD and KJ regenerative processes. In total,32 rabbits were used in studiesA-C.A) IVD cells were sorted; FACS by size (forward scatter; ≤10µm or >10µm or GDF5+cells(anti-GDF5 antibody). Sorted cells, with celltracer were applied on IVD-explants in vitro. Migrating cells/distance was evaluated by fluorescence-and-confocal-microscopy(FC).B) DNA-labeling was performed with BrdU(oral ad). Animals were sacrificed(14-56 days), KJ collected and BrdU+cells visualized by IHC /anti-BrdU-antibody in SN and articular cartilage(AC).C)Cell tracers(iron-nanoparticles/fluorochrome;CFDA) were injected to SN of IVDs(LI-LV) and KJs(tibia).Animals were sacrificed(2-6 weeks). Labelled cells traced by ferric-iron-staining and FC. Results:A)GDF5+cells and ≤10µm cells displayed best migration capability in IVD explants. GDF5+cells were detected at tissue depth 1300µm(16 days).B)BrdU+cells were observed in early time points in SN of KJ, at later time points in AC, indicating a gradual migration of cells.C)Fe+cells were detected in IVDs >1200 µm from injection-site in all animals. In KJ, Fe+cells were detected in 56% of the animals in AC, results supported by FC. Results indicate similar cellular migration patterns in cartilage (IVD and KJ). This is of interest from a tissue engineering perspective.
Synergistic Effect of Adipose Stem Cells and Bone Marrow Stem Cell to Promote Osteogenic Differentiation and Angiogenesis

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The purpose of this study was to determine the synergistic effect and the optimal ratio of adipose stem cells (ASCs) added to BMSCs (bone marrow stem cells) to enhance the osteogenic differentiation and angiogenesis. In vitro segregated cocultures using transwell were carried out for 14 days using 1 x 10^5 BMSCs and ASCs of variable number (0.25 x 10^5, 0.5 x 10^5, 0.75 x 10^5, and 1 x 10^5) cells. In vitro mixed cocultures were also performed in the same proportion. For in vivo analysis, cells were seeded in PLGA scaffold and implanted on the subcutaneous tissue of 20 nude mice in following 4 ways and analyzed after 5 weeks: 1) no cells; 2) 1 x 10^5 BMSCs; 3) 1 x 10^5 BMSCs and 0.5 x 10^5 ASCs; and 4) 1 x 10^5 ASCs. From the transwell culture, 1 x 10^5 BMSCs cultured with 5 x 10^5 ASCs showed significantly greater osteogenic differentiation and mineralization as shown by alkaline phosphatase (ALP) activity and calcium deposition than BMSCs alone. In the mixed coculture model, ASC/BMSC coculture at a ratio of 0.5/1 showed a significantly greater level of ALP activity and calcium deposition as well as greater gene and protein expression of osteogenic markers than BMSCs alone. The mixed ASC/BMSC coculture at a ratio of 0.5/1 showed the highest level of vascular endothelial growth factor and the most effective tube formation from human umbilical vein endothelial cells. In vivo implantation studies demonstrated that PLGA-ASCs-BMSCs had number of vascular structures significantly greater than PLGA-BMSCs, and the calcification greater than PLGA-ASCs. In conclusion, ASCs added to BMSCs promoted osteogenesis and angiogenesis with the optimal ASCs/BMSCs ratio of 0.5/1.

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Morphologic Assessment of Polycaprolactone Scaffolds for Tracheal Transplantation in a Rabbit Model

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The purpose of this study was to clarify whether a polycaprolactone (PCL)-framed porous tracheal scaffold could be used for the replacement of the trachea in rabbits and produce better results in terms of luminal epithelialization. The tracheal scaffold consists of a PCL framework and a collagen layer. After a longitudinal cervical skin incision, the trachea was exposed and a rectangular defect (1 x 0.5 cm) was created on the cervical trachea by a scalpel on eight rabbits. PCL-scaffold was trimmed and fixed to defect boundaries with Tisseel. Postoperatively, the site was evaluated endoscopically and histologically. Bronchoscopic examinations at 1 week revealed that implant exposure was recognized in the entire length of the prosthesis. The luminal surface of the implanted scaffold was partially covered at 2 weeks and completely covered at 4 weeks. Histologic data showed that the epithelial lining was nearly completed 1 week after surgery and some inflammatory cells were seen in the submucosa. At 2 weeks, the epithelium was already covered and the migration of inflammatory cells was not observed. However the concentration of cilia was not observed at this week. At 8 weeks there was also a neovascularization with luminal epithelialization. These findings suggest that a PCL-framed porous tracheal scaffold used in our experiment is an effective way to regenerate the epithelium on the surface of an artificial trachea.
An In-vitro Model of Diabetes and Obesity

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Nutrient metabolism in humans is a complex process co-ordinated by two key compartments, hepatic and adipose tissue. The vascular system provides a communication link between the two, for exchange of both metabolites and signaling molecules. Presently, metabolic coordination between organs and tissues is investigated using animal models, some designed to manifest symptoms of metabolic diseases such as obesity and insulin resistance. As these models are poorly representative of human responses, there is a clear need for more physiologically relevant models using human cells and tissues. To this end we have developed an in-vitro model of nutrient metabolism, recreating the hepatic, adipose and vascular microenvironment through the use of bioreactors and 3D scaffolds and connecting the tissues together in a fluidic system representative of blood circulation through organs. Since overnutrition and its complications are major health issues, our aim is to develop disease models of diabetes and obesity by combining the tissues in different proportions representing normoweight, overweight and obese. The vascular compartment is represented by human endothelial cells plated in high shear stress bioreactors. The hepatic compartment consists of the human hepatic cells seeded on 3D collagen scaffolds, and the adipose compartment is composed of human omental fat in different proportions. The three tissues were studied singly, and then in combinations of 2 and 3, in media with different glucose levels. The compartments were analyzed for metabolic, stress and morphological markers. Our results show that the system can recapitulate the main features of human metabolism, as well as stress and inflammation associated with overnutrition.
Membrane Bioreactor for Expansion and Differentiation of Embryonic Liver Cells

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There is a growing demand for the expansion and differentiation of stem cells for cell therapies, tissue engineering, and model systems for drug screening. Current methods for stem cell production are based on the use of batch tissue culture flasks, which have several drawbacks. In this paper we report on the use of a crossed hollow fiber membrane bioreactor for the expansion and differentiation of embryonic liver cells, which have been used as an alternative model of human liver progenitor cells. The bioreactor consists of two bundles of hollow fiber (HF) membranes with different molecular weight (MW) cut-off and physico-chemical properties cross-assembled in alternating manner: modified polyetheretherketone (PEEK-WC) and polyethersulfone (PES), which perform different functions. PEEK-WC HF membranes provide cells nutrients and metabolites whereas PES HF removes catabolites from cell compartment mimicking in this way the in vivo arterious and venous blood vessels. The combination of these two fiber sets creates three compartments: two intraluminal compartments of PEEK-WC HF and PES HF in which the medium flows and one extraluminal compartment represented by extracapillary network formed on both type of fibers in which cells are cultured. This bioreactor geometry ensures high mass exchange of nutrients and metabolites, which were important for cell proliferation and differentiation. Embryonic liver cells grown on and around the fibers in the crossed HF membrane bioreactor increasing their density of 97%. The membrane bioreactor promoted the functional differentiation of cells inducing the expression of the liver specific metabolic functions in terms of urea synthesis, albumin production and diazepam drug biotransformation.
Biomimicking Hyaline Cartilage Microenvironment with an Innovative Bioreactor System

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Hyaline cartilage cells, the chondrocytes, have an in vivo microenvironment characterized by mechanical stimulation, shear stress, limited oxygen and nutrient supply. Biomimicking this milieu was the aim of the development of a bioreactor system (BRS). All experiments were performed with primary chondrocytes, isolated from articular cartilage of freshly slaughtered pigs by digestion with collagenase-A (2mg/ml) for 16 hours. The obtained cell suspension was mixed with prewarmed agarose type VII solution with a final cell concentration of $4.7 \times 10^5$ cells per gel (agarose, 3% v/v). The custom-made, autoclavable, modular BRS contained six positioning stages for the parallel cultivation of chondrocyte-seeded agarose gels (each Ø10 x 3mm). Despite the application of shear stress with a peristaltic pump (47 or 94µl/min), the cells were stimulated by sinusoidal or trapezoidal mechanical loading pattern (1Hz, 15% strain, 5min on / 235 min off) over 14 days of cultivation at 5 or 20% oxygen. The BRS showed a significantly increased production of sulfated glycosaminoglycans per cell due to minimal proliferation and enhanced extracellular matrix secretion compared to cell-seeded gels, cultivated in petri dishes. In summary, the BRS was capable of biomimicking the microenvironment of articular cartilage in an agarose gel model. Further experiments will focus on different scaffolds and the approximation to even more in vivo-like stimulations of chondrocytes-seeded matrices.
Advanced Bioreactor Technologies to Provide Optimal Culture Conditions In vitro

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In Tissue Engineering, one of the most challenging tasks for the generation of functional tissues is to guide tissue development in vitro. When growing a tissue construct without physiological stimuli, the cells stop producing tissue specific extracellular matrix or start to dedifferentiate into tissue unspecific cell types. Therefore, physiological cues equal to the in vivo situation are needed. In our studies, we developed tailor made bioreactor systems for four (bone, skin, bloodvessel and planar reactors) tissue engineering applications. In addition, the different bioreactors can be used in combination with a self-engineered incubation camber. Strictly defined physiological parameters e.g. gas concentration, temperature, pH, perfusion and mechanical pressure ensure reproducibility and robust culture conditions. This incubator technology reduces manual handling and allows a continuous monitoring of the experiment. To show our results, we want to give an example each for soft and hard tissues. For our application in muscoskeletal applications, we seeded the cells inside of the scaffold followed by a 2-4 week maturation phase inside of the bioreactor. The gene expression for osteogenic differentiation was measured and it could be shown, introducing controlled perfusion and mechanical stimulation in our incubator systems activates the expression of explicit differentiation markers. In soft tissue applications, we are able to create skin equivalents mimicking the native skin. There are remaining only slight differences in comparison of the histological stainings. With implementation of other cell types, the accuracy of the skin model can be further extended.
Co-cultures in the Hollow Fiber Bioreactor: A Good Platform for *ex vivo* Expansion of Hematopoietic Stem Cells?

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Over decades, the hematopoietic stem cell (HSC) niche has drawn great attention because of its importance in regulating HSC functions such as quiescence, self-renewal and differentiation. The bone marrow microenvironment, constituting stromal cells and the extracellular matrix (ECM), is crucial for the maintenance of hematopoiesis *in vivo*; while *ex vivo* it can be used to manipulate HSCs. Many stromal supported HSC co-culture systems, developed by various groups, confirmed that stromal cells play an integral role in HSC fate decisions such as enhancing HSC proliferation and self renewal *in vitro*. *Ex vivo* expansion of HSCs has high clinical relevance and many groups have developed bioreactor systems that support large-scale *ex vivo* expansion. In this study, we developed a co-culture platform based on the hollow fiber bioreactor (HFBR) for *ex vivo* expansion of cord blood derived HSCs. We outlined a cell culture methodology for establishing stroma-supported serum-free *ex vivo* expansion culture using a human bone marrow stromal cell line HS-5. We also evaluated the efficacy of such a culture platform in comparison to standard flask cultures in supporting HSC proliferation and preserve HSC stemness. Our study showed that the HFBR was better at supporting expansion of HSC progenitors while preserving its clonogenic ability. Moreover, the HFBR can reach a larger scale cell culture in a small volume eliminating the need for multiple tissue flasks to support *ex vivo* expansion. Interaction between HSCs and stromal cells is significantly enhanced in the HFBR due to its three-dimensional cell-matrix architecture. We conclude that the HFBR system recreates a closer mimicry of cell-stroma microenvironment, which can provide significant insights on *in vitro* studies of stroma-dependent hematopoietic cell functions. These findings open up the feasibility of utilizing the HFBR as a good *in vitro* mimicry of the human bone marrow for large scale expansion of HSCs.
The effect of fluid flow shear stress on surface modified silicone hollow fibers with the aim of designing a biohybrid artificial lung was investigated in this paper. Silicone fibers were surface modified with radio frequency glow discharge (RFGD) and collagen adsorption to increase Human Umbilical Vein Endothelial Cells attachment, growth and stability. The fibers were placed in the parallel plate flow chamber and exposed to different shear rates using a peristaltic pump. Ten silicone hollow fibers were treated RFGD (frequency of 40 kHz, an oxygen flow rate of 50 sccm, a power of 60 W and the plasma treatment time of 120 s). After that, the samples were soaked in collagen type I for protein immobilization. 10^5 HUVECs in 10ml of culture medium were added to the fiber containing tube and placed on a rotor overnight. These cell seeded fibers were fixed on a slide glass were inserted into a novel parallel plate flow chamber. DMEM without any additive was circulated through this chamber with a pump at 25-40 ml/min for 1 hour.

The loading of hydrodynamic mediate shear force for 1 hour to the surface modified hollow fibers coated with endothelial cells had little effect on the density of adhered cells.
A Microfluidic Platform for Drug Testing in the Fourth Dimension

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Investigation of cell-drug interaction is of great importance in drug testing but poses great challenges to develop robust and truly predictive methods. Microfluidic bioreactors allowing organotypic 3D cultures have emerged as a promising technology, and their miniaturization and flow-through operation significantly reduce sample consumption while improving the test reliability and resolution. We present a new microfluidics-based platform suitable for optical interrogation of long-term 3D (i.e. 4D) organotypic cultures. The system was designed based on multiphysics simulation of culture medium flow and oxygen mass transport. It consists of a set of transparent chambers hosting cell/scaffold constructs, of length 6 mm, width 3 mm, and thickness 400 µm. The medium flows through microfluidic channels by a syringe pump. Live optical diagnostics in 3D and oxygen tension measurement are made possible in each chamber. To demonstrate the ability of our system to provide stable, long-term maintenance of high-density cellular constructs, we observed the growth of MG63 human osteosarcoma cells cultured under 3D conditions up to 7 days. Cell viability was well maintained and cells intensely proliferated and synthesised matrix. On live constructs, cell proliferation and scaffold pore occlusion were successfully measured through optical interrogation. The oxygen tension progressive drop, due to increased cellularity, was in accord with the one predicted by multiphysics computation. We now plan to apply the system to test anti-Parkinson drugs on organotypic cultures of dopaminergic neurons, and also to test non-viral gene delivery vectors on organotypic cultures of solid tumors.
Reconstruction of Human Articular Cartilage in Collagen Scaffolds under Bi-Directional Perfusion

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Cartilage has poor capacity for self-repair and tissue engineering is an alternative strategy to restore functional cartilage tissue. Besides, the recent developments in perfusion culture technology offer interesting possibilities. Here, we developed a tissue engineering procedure combining human articular chondrocytes (HAC), collagen sponges and the use of a perfusion bioreactor prototype. We previously reported the enhanced production of cartilage characteristic matrix in collagen sponges in the presence of a growth factor cocktail consisting of BMP2, insulin and the thyroid hormone T3 (BIT). The addition of bi-directional interstitial perfusion to this cocktail aims at improving the quality and uniformity of the synthesized matrix, so as to obtain a more functional implant. After 3 weeks of amplification, HAC were seeded in collagen sponges by using a prototype of OPB (Oscillating Perfusion Bioreactor). The constructs were then cultivated for 21 days under bi-directional perfusion. We established a perfusion speed program based on phases of high and low perfusion speeds to allow alternation of HAC stimulation and matrix deposition. The chondrocyte phenotype and the matrix production and deposition in collagen sponges were evaluated by real time PCR, Western Blotting and immunohistochemistry analyses. To further estimate the stability of the neo-synthesized matrix, the constructs were implanted subcutaneously in nude mice for 6 weeks. The results show that perfusion allows homogenous deposition of a hyaline-like cartilage matrix within the sponges. Interestingly, a much lower amount of type I collagen was produced in dynamic culture conditions when compared with static conditions, therefore limiting the risk of fibrocartilage production.
In this work we present the use of biomimetic bioreactors imitating physiological environments in skeletal tissues for functional evaluation of biomaterials with the special focus on the design and validation of a novel bioreactor with hydrostatic pressures. In specific, we have utilized perfusion bioreactors and a bioreactor with dynamic compression for studies of nanocomposite alginate discs containing silver nanoparticles attractive as potential antimicrobial cell scaffolds, tissue implants, and wound dressings. Mechanical stability of the discs and release kinetics of silver nanoparticles and/or ions were assessed under continuous perfusion of saline solution imitating physiological conditions in vascularized tissues (20–50 µm/s fluid velocities) as well as under dynamic compression (0.1-1 Hz compression frequency, 10 % strain, 1 h on/ 1 h off) imitating mechanical loads imposed on articular cartilage in vivo. In conjunction with these studies, a novel bioreactor providing hydrostatic pressures up to 30 bar and continuous medium flow during non-pressurized phases was examined with respect to pressure control and flow patterns with the aim to establish conditions relevant for intervertebral discs. This bioreactor was further used to reveal the effects of hydrostatic pressures on nanocomposite alginate discs. Based on results of these studies we are developing models that would reliably predict biomaterial behavior upon implantation in vivo.
Biofabrication and Dynamic Culture of Micro-Vascularized Constructs Based on Fibrin and MSCs for Bone Tissue Engineering

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Engineered bone substitutes are emerging as a potential therapeutic alternative to autologous bone grafts, although the absence of functional vascularization at the time of implant is a significant drawback. We already reported the generation of vascular structures by endothelial monolayers transfer from rods to hydrogels. The aim of this work is to produce pre-vascularized fibrin gels with embedded mesenchymal cells (MSCs) as candidate bone substitutes. HUVECs were seeded on gold rods coated with a SAM-forming oligopeptide and transferred electrochemically to fibrin gels with MSCs or MSCs+HUVECs or w/o cells. Constructs were cultured in a perfusion bioreactor for 7 days. Aiming at fabricating constructs with clinically relevant dimensions (6.5 mm Ø, 8 mm thickness) we hypothesized the need of multi-channeled constructs. To optimize their geometrical configuration we performed computational oxygen distribution analyses, which were then correlated to a simplified experimental 3D model for preliminary cell behavior screenings. Successful transfer of intact HUVEC monolayers to fibrin was obtained and the vascularized channel maintained its structure and cell viability during perfusion culture in bioreactor. After 7 days endothelial sproutings from the channel in the gel were observed mainly in the presence of MSCs and HUVECs embedded. Simulations showed that complete oxygenation was achieved with at least 3 channels. Screenings on the simplified model showed new microvessels sproutings between HUVEC monolayers. In conclusion, constructs with geometrically controlled micro-vascularization can be generated based on fibrin gels loaded with MSCs and cultured in a bioreactor, in view of vascularized bone grafts engineering.
An Ex Vivo Approach for Studying the Pressure-Driven Early Remodeling of the Human Saphenous Vein in Vein Graft Disease

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Saphenous vein (SV) graft disease represents an unresolved problem in coronary artery bypass grafting (CABG). Altered wall strain consequent to transplantation in arterial position is supposed to play a pivotal role in pathology initiation and progression. The aim of this study was to develop an ex vivo approach to expose SV segments to controlled arterial-like wall strain and study its global effects on the arterialization process. An ex vivo vessel culture platform able to apply the desired pressure patterns to SV segments - 80-120 mmHg for CABG-like pressure (CABG) and 5 mmHg constant pressure for vein-like stimulation (VP) - was designed and manufactured. Human SV segments were then cultured for 7 days under CABG or VP conditions. Morphological analyses performed onto the CABG vs. VP-stimulated segments showed that arterial-like pressure condition determined a major reorganization of the vessel wall components. This included partial endothelial denudation, smooth muscle cells disarray and a rearrangement in vasa vasorum. Further, a decrease of SV wall thickness and enlargement of the luminal perimeter were observed. As expected, it was also found an increased proliferation rate in cells in the SV wall and activation of MMP-2. These results show the ability of the ex vivo SV culture platform to reproduce the effects of altered wall strain in SV arterialization. These data provide a new basis for future molecular studies addressing CABG failure.
A Bottom-up Approach to Engineer Bone Grafts with hESC-MSC-laden Microcarriers

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Modular tissue engineering (TE) is promising to overcome the limits in traditional TE. Human embryonic derived mesenchymal stem cell (hESC-MSC)-laden microcarriers can be used as building blocks that can assemble and create large bone grafts. The bone forming capacity of hESC-MSC in a bottom-up approach was never studied and compared with classical top-down approaches. In the present study, the feasibility of hESC-MSC seeded on microcarriers (bottom-up) was compared with scaffolds (top-down) to form bone TE grafts. hESC-MSC were derived by standard monolayer derivation and immunophenotypically characterized by flow cytometry (CD73⁺, CD90⁺, CD105⁺, CD34⁻, CD45⁻, CD20⁻, CD14⁻) followed by a 2D osteogenic differentiation assay. For 3D analysis, cells were seeded on collagen-based microcarriers/scaffolds and cultured in dynamic conditions for 4 weeks. The bone-like morphology and expression was evaluated by (immuno) histochemistry and gene expression (Runx2, collagen I, BSP, osteocalcin). In the top-down approach, scaffolds were colonized, although the center of the scaffolds was never completely colonized. In the bottom-up approach, cells remained viable throughout the culture period. Aggregate formation was observed already after 7-14 days. From 14-28 days, aggregates (microtissues) were clustering together by self-assembly and were forming macrotissues. A uniform cell distribution, high cellularity, high density and dense extracellular matrix formation could be achieved in the center of the macrotissues. This study reveals the potential of hESC-MSC-laden microcarriers as building blocks to build up macrotissues in a bottom-up approach.
The production of cardiomyocytes (CM) from induced pluripotent stem cells (iPSC) holds great promise for autologous regeneration therapies, patient-specific disease modeling and cardiotoxicity testing. However, the widespread clinical use of these cells is still hampered by the lack of robust bioprocesses for the production of CM in high purity, consistent quality and relevant quantities. The main goal of this study was to develop a scalable platform for the production of pure iPSC-derived CM. Our strategy consisted in designing an integrated bioprocess for CM differentiation and purification in environmentally controlled bioreactors, where the necessary conditions to control stem cell fate are thoroughly tuned.

A transgenic murine iPSC line, in which the cardiac-restricted α-myosin heavy chain promoter drives both eGFP and puromycin resistance gene expression, was used. iPSC were cultivated as aggregates in distinct bioreactor systems (stirred tank and Wave™ bioreactors) and the impact of cyclic mechanical strains, promoted by different stirring profiles, on CM differentiation was evaluated. Our results showed that stirring is a key parameter in iPSC differentiation towards functional CM. Using an intermittent stirring profile in stirred tank bioreactors, we were able to improve by 1000-fold the differentiation yields (up to 43 CM per initial iPSC) and the final CM purity (>97%). Furthermore, we showed for the first time that wave-induced agitation of Cellbag™ bioreactors enhances CM differentiation. CM-committed cells were identified earlier and a higher differentiation yield was achieved (60 CM per initial iPSC) when compared to stirred tank bioreactor cultures.
Extrinsic Vascularisation Leads to a Faster Vascularisation and Bone Formation of an Axially Perfused Tissue Engineered Large Volume Bone Substitute

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Reconstruction of extensive bone defects is still a great therapeutic challenge. In the past few years, the concept of tissue engineering using axial vascularisation was established in order to generate transplantable, vascularised large volume constructs in the sheep arteriovenous (AV) loop model. However, generating such a bone substitute which is mechanically stable enough for transplantation in a critical size bone defect has yet not been realised.

In this study, a nanostructured bone graft material was evaluated in combination with autologous blood in the sheep AV-loop model using a closed teflon or a perforated titanium isolation chamber. Bone formation and vascularisation were monitored by intravital imaging, immunohistochemistry and molecular biology methods. Constructs were explanted up to 18 weeks. Using the perforated titanium chamber additional extrinsic vascularisation supporting the newly developed tissue inside the chamber was visualized by sequential perfusion of the explants. An accelerated vascularisation of the constructs inside the perforated chamber could be shown over time by recruitment of the former extrinsic vessels which connected to the intrinsic (axial) vascularisation axis. This earlier vascularisation led to increased remodeling and earlier bone formation compared to the closed teflon chamber. Immunohistochemical and real time-qPCR analyses proved the expression of angiogenic and osteogenic genes.

The next step will be the transplantation of an engineered bone block in the sheep AV-loop model into a critical size tibia defect. In the future this concept could possibly replace current therapeutic concepts for treatment of bone defects.
A Custom Made Perfusion Bioreactor System for The Cultivation of Large Scaled Scaffolds - Spatial Distribution, Differentiation And Oxygen Consumption of Mesenchymal Stromal Cells

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Due to the high socioeconomic relevance of musculoskeletal disorders innovative concepts for bone regeneration are essential. In this context, a major prerequisite is the dynamic cultivation of three-dimensional porous cell-scaffold constructs in a clinically relevant size. The aim of the study was to develop a perfusion bioreactor system suitable for the dynamic cultivation of high-volume cell-scaffold constructs. Tricalciumphosphate cylinders (20 mm x 25 mm) were seeded with 6.3 x 10\textsuperscript{6} immortalized human mesenchymal stromal cells (MSCs) and cultured for 14 days in a modular, custom designed perfusion bioreactor system with integrated online oxygen measurement. Constructs cultured under static conditions served as control. Cell distribution and vitality in the longitudinal central plane were examined after 1, 7 and 14 days using confocal laser microscopy. Cell proliferation was assessed by lactate dehydrogenase content whereas oxygen concentration was monitored online in the center of the scaffold and at the in- and outlet of the bioreactor chamber. After 7 days no significant differences concerning cell proliferation could be observed between static and perfusion culture. After 14 days statically cultured cell-scaffold constructs showed huge necrotic zones in the center due to a critical drop in oxygen concentration. In contrast, oxygen levels within the perfused scaffolds remained stable throughout the whole culture period that allowed the cells to stay viable even in the center of the construct. From this we conclude that perfusion is essential in high-volume cell-scaffold constructs. Notably perfusion in large scale has to be optimized in up scaled scaffold sizes to enhance cell proliferation and differentiation.
Definition of Standards for the In vitro Fabrication Process of Tissue Engineered Human Heart Valves

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Currently there is no “gold standard” for the fabrication of human heart valves using tissue engineering methods: each tissue engineered heart valve is individually fabricated. For future clinical applications the fabrication process, including cell isolation, expansion and seeding, scaffold fabrication and afterwards the conditioning process in specific bioreactor systems, needs to be standardized and controlled in each stage of the process. We have developed a new, GMP-conform pulsatile bioreactor system with an integrated measurement unit to collect data via sensors for O₂, CO₂, pH, temperature and medium circulation. Furthermore, medium samples can be taken during cultivation for analysis of extracellular matrix components, inflammation markers and other segregated proteins. Cell-seeded heart valve scaffolds can be cultured under standardized conditions with complex accompanying analytical procedures. Compared to conventional bioreactors the new pulsatile bioreactor offered the possibility of using strongly varying pulsation in order to determine the optimal conditions for tissue generation. The selected parameters O₂, CO₂, pH, temperature, circulation and composition of the culture medium during conditioning in the bioreactor system served as quality controls and prerequisites for in vivo testing. Definition of standards for the fabrication process of tissue engineered human heart valves is of particular importance for their eventual recognition as an advanced medicinal therapy product (ATMP). Therefore the single steps of the whole fabrication process have to be carefully evaluated and standardized in accordance with existing international standards (ISO).
Galectin-3 and Collagen IV Rescue Tubular-like Structure Formation under Dynamic Conditions in a Bioreactor System

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Nutrient and oxygen supply are essential for an engineered tissue with more than 100-200µm of thickness, which can be ensured by blood vessels. However, formation of tubular-like structures in a co-culture system of human foreskin fibroblasts (HFFs) and human umbilical vein endothelial cells (HUVECs) under dynamic conditions has not been investigated yet. Additionally, the effect of galectin-3 and collagen IV in this system exposed to flow is examined. The HFF/HUVEC co-culture system has been moulded in fibrin gel and cultivated for 9-21 days under static conditions. In comparison, 14 days of dynamic co-culture was performed using a custom-made bioreactor system. Furthermore, the effect of galectin-3 (10µg/ml) and collagen IV (30µg/ml) on the formation of tubular-like structures has been investigated. After 9-21 days of cultivation, gels were fixed and immunostained (CD31). Visualization of tubular-like structures in the 3D fibrin matrix was carried out using two-photon microscopy and ImagePro® Analyzer software. The HFF/HUVEC co-culture system under dynamic conditions led to less tubular-like structures compared to static conditions. Addition of galectin-3 and collagen IV rescued the tubular-like structure formation under dynamic conditions.
A Novel Prevascularized Fibrin-based Organotypic Skin Construct

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Improvements of organotypic skin constructs (OtSC) are deemed necessary for widespread use in regenerative medicine. Thus, our studies took a three-pronged approach to modify existing concepts in skin tissue engineering: 1. Prevascularization to improve take rate of OtSC and possibly their clinical outcome. 2. Use of Bioreactor technology to simplify and optimize handling procedures of OtSCs. 3. Investigation of a possible complete autologous method by using a fibrin scaffold in vitro. A custom bioreactor was designed for the use with off-the-shelf cell culture inserts. Human OtSCs consisting of foreskin keratinocytes (HuFK), foreskin dermal fibroblasts (HuDeF) and umbilical vein endothelial cells (HuVEC) were molded with fibronectin-coated fibrin gel as a scaffold. After up to 14 days, OTSCs were evaluated using HuVEC and skin development markers by 2D immunostaining. The influence of different flow conditions and HuFK seeding densities on capillary-like structure (ClS) network formation was analyzed by two-photon microscopy and ImagePro® software. To our knowledge, Bioreactor supported construction of prevascularized fibrin-based OTSCs was experimentally realized for the first time. However, the number of capillary-like structures decreased with higher HuFK seeding densities and high flow rates in the Bioreactor. Branching, lumen formation and diameter of the ClS are additionally modified by the HuFK seeding. Our results hint at a complex interdependency of epithelial formation, angiogenesis and culture conditions. The inclusion of ClS in our autologous OtSCs is promising for their future use in R&D and could well improve their clinical performance.
Modulation of Mesenchymal Stem Cells Secretome by Bioreactors Increases Neuronal Survival and Differentiation of Human Neural Progenitors

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Human Mesenchymal stem cells (hMSCs) have been proposed as possible therapeutic agents for central nervous system (CNS) disorders. Nowadays it is suggested that their effects are mostly mediated through their secretome, which contains several neuroregulatory molecules capable of increasing cell proliferation, differentiation and survival into different physiological conditions. Additionally, as MSCs are highly responsive to dynamic culturing environments, one could expect to modulate and possibly increase the level of the above referred neuroregulatory factors in the secretome through the use of bioreactors. Therefore, in the present work we aimed to assess if the use of a dynamic culturing condition was able to modulate the secretome of BM_MSCs, and if this secretome was able to modulated neuronal/glial cell survival and differentiation of human telencephalon neural progenitors (htNPCs) \textit{in vitro} when compared with the static condition. The results revealed that the use of a bioreactor system modulates the secretome of BM-MSCs once, when incubated with htNPCs high levels of survival and differentiation into newborn neurons (DCX$^+$), and mature neurons (MAP-2$^+$) with GABAergic phenotype (Gaba$^+$) were obtained when compared with the secretome from the static condition. Currently we are analyzing the effects of this dynamic secretome in an \textit{in vivo} approach but so far, with this work it is possible to show that MSCs seems to be highly responsive to dynamic culturing environments, increasing the neuronal survival and differentiation of neuronal progenitors \textit{in vitro}. This might be of relevance for the future applications of these cells/secretome in CNS related therapies.
Effect of Shear-stress on Protein Secretion in Tyrosine Hydroxylase (TH) Positive Cells

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Mesenchymal stem cells (MSCs) secrete a substantial variety of proteins to the microenvironment that constantly reshape the extracellular matrix composition. These proteins are also important means of MSC communication. The secretion of protein is therefore very critical regulating the cellular progresses and determining their functionality. Tremendous amount of data was collected so far on protein secretion by static culture. However, the biological systems are dynamic and mostly involve a flow, which apply a shear force to the cells. For that reason, the analysis of shear stress effect on MSCs is very important. In this study, the cells were fixed on a collagen coated solid scaffold, ie. glass and polymethyl methacrylate (PMMA), and the cells were cultured in a temperature controlled packed-bed bioreactor for up to 10 days by continuous-flow perfusion system. Bone marrow derived MSCs were used to obtain stable recombinant cell lines expressing TH ectopically that eliminates any effect of endogenous control. The analyses showed that the flow under constant atmospheric pressure increase the protein secretion levels regardless of the type of the support (0.64 ng/ml). Continuous flow had significant effect on secretion, as much as twice of those measured in static culture. Remarkably, the reduction in protein secretion was estimated under high shear stress conditions (0.28-0.33 N/m²) compared to low stresses (0.15-0.20 N/m²). Although both scaffolds were coated with collagen, PMMA showed higher collagen coating levels and higher TH levels. Using a bioreactor, the physiological conditions were simulated, and the significant effect of shear stress was demonstrated.

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Compressed Collagen Scaffolds for Enhancing Urinary Tissue Regeneration in a Bioreactor Cell Culture System

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Tissue engineering of urinary tract plays a pioneering role among many branches of tissue engineering. When the surgical repair of the urinary tract is required, the techniques of the reconstructive procedures have several drawbacks such as secondary malignancies, intestinal adhesions, and chronic infections. Thus the latest developments in urinary tract tissue engineering have been aiming to design novel culture techniques. The use of bioreactors in tissue engineering is regarded as a further step in comparison with conventional tissue engineering techniques. In this study, after determining the optimum flow parameters for the system, compressed collagen tubes were assessed under dynamic culture conditions inside a novel bioreactor system imitating the dynamic environment similar to the ureter. In vitro biocompatibilities via smooth muscle and urothelial cells of these engineered compressed collagen tubes have been investigated. Currently biological performance of the compressed collagen tubes is being evaluated by AlamarBlue assay and immunohistochemistry.
Computational Fluid Dynamics Model to Optimize Air-Lift Bioreactor Geometry for Stem Cell Expansion

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Air-lift bioreactors can play an important role in tissue engineering because of many advantages, such as; simplicity of construction, absence of moving parts, easy sterilization and low power consumption. However, optimum design of airlift bioreactors for cultivation of stem cells is still a difficult task, because of complicated relations between mixing and mass transfer, and also high sensitivity of stem cells to shear stress. In this study, computational fluid dynamics (CFD) analysis using related software was used to characterize an internal loop airlift bioreactor for stem cell proliferation. The effect of geometry parameters such as sparger position and configuration, ratio of the downcomer to the riser area (Ad/Ar), and height of liquid above (Ha) and below (Hb) of the draft tube was considered to identify the complex hydrodynamic environment as well as shear stress applied to suspension culture of stem cells. The results demonstrated that sparger position and configuration has maximum effect on shear stress exposure on cell although shear stress can be reduced with increasing Ha in this area. In addition, large Ad/Ar was found to be useful for minimum exposure shear stress on cells while it has negative effect on cell distribution as same as Hb . Therefore, optimum design of air-lift bioreactor can be achieved by CFD analyzing with comprehensive considering of hydrodynamic environment to apply this type of bioreactor in tissue engineering without performing costly time-consuming experiments.
Bioreactor for Dynamic Biological Barriers

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One of the specifications required for simulating biological barriers is to create an interface between two fluid phases in which cells can be seeded. It can be an air-liquid interface (lung, skin) or a liquid-liquid interface (intestinal epithelia). The standard system used to mimic these barriers is the transwell insert in static culture conditions, but it is well known that the physiological environment is characterized by dynamic conditions due to the presence of flow and continuous motion (breathing, peristalsis etc.). Here a new bioreactor which can combine a transwell-like cell culture with medium flow rate is presented: the Membrane Bioreactor (MB). It is made in Poly-dimethylsiloxane, a biocompatible autoclavable polymer with self sealing properties. With respect to the MCmB (MultiCompartment modular Bioreactor, now commercialized as Quasi-Vivo), the MB includes an innovative holder which houses a commercial porous membrane, providing a good support for cell seeding and improving the usability of flexible membranes. The holder with the membranes represents an alternative to the traditional transwell, but it can be easily inserted in a flow bioreactor to perform dynamic cell cultures. Moreover, trans epithelial electrical resistance (TEER) measurements can be performed using the EVOM2 (World Precision Instrument Inc.), to monitor barrier integrity. Once cells reach confluence, the holder can be placed into a fluidic chamber composed by an apical and a basal compartment with inlets and outlets, to assure media flow and mimic the dynamic physiological conditions. The fluid dynamics inside the bioreactor was characterized using a CFD model, while the inner pressure and the exchange of solutes through the membrane using in-vitro tests. To demonstrate the advantages of the dynamic intestinal barrier model, Caco-2 cells were cultured in the MB and cell vitality, TEER and tight junction formation were compared with cells in static transwells.
Developing Lab Equipment with Healthcare Researchers: 
A Participatory Design Analysis

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In this paper, we explore how a participatory design approach can be applied to scientific research, in the field of regenerative medicine. Though user-centred practices are employed to support patient-side activities, not as much has been done to investigate how different design methodologies can engage researchers, being a crucial workforce for medicine. We compare case studies taken from history with an experiment concerning the design of an Oscillating Perfusion Bioreactor, a bio-medical device now being used as a research platform destined to tissue engineering and regeneration. Taking autologous cells, it performs 18 parallel cell culture threads, in a multi-array of confined, controlled chambers. Our own product design intervention started from the perfusion patent and an early prototype, presenting several issues in weight, scalability, kinematics and affordance. How can designers intervene into complex research to evolve its equipment? How can industrial design get involved as a structured discipline into the development of science? What can designers exactly improve in the performance of the final product? How can design research support research in science? Our research methodology concerns a step-by-step analysis of both the (evolving) research environment and healthcare researchers, taking into account their habits, expectancies and cultural schemes, providing clues for engaging better participatory design practices, both in terms of final product qualities and time-effectiveness. The findings provide a detailed theoretical position concerning the disciplinary code of industrial design and practical indications for taking better policies when applying design management to healthcare research.
In Situ Enhancement of Mandibular Regeneration and Vascularization Through Microvascular Techniques

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Most of the current regenerative medicine approaches for in situ regeneration rely on extrinsic vascularization of grafts. This could not be applied to reconstruction after cancer ablation and irradiation because of the resulting hypocellularity and hypoxic local environment. The study aimed at investigating the efficacy of inducing axial vascularization of scaffolds to regenerate critical size mandibular defects in situ.

The study is a controlled comparative experimental study performed on two groups of animals each included 4 adult male goats with critical size marginal mandibular defects. The defects were reconstructed with biphasic ceramic scaffolds charged with BMP2 and PRP. Only in the group A an arteriovenous loop (AVL) was created through micro-anastomosis of facial vessels and set inside a special groove in the scaffold. After 6 months the mandibles were examined for bone regeneration and angiogenesis through computerized topographic angiography (CTA), histological and histomorphometric analysis. The biomechanical properties of the mandibles were assessed through a 3 points bending test.

Group A showed better vascularization and bone formation. Vascularization of the central regions of the scaffolds was significantly higher as evidenced by histomorphometric analysis (p value 0.0214). The increase in bone formation was, however, not statistically significant (p value 0.0821). Although the biomechanical integrity was recovered in comparison with mandibles with bare defects, there was no statistically significant difference in the biomechanical properties between group A and B.

In situ induction of axial vascularization of synthetic mandibular grafts leads to enhanced vascularization at their central parts permitting better in situ tissue regeneration. This can be of significance in cases of large irradiated bone defects.
Non-Invasive On-Line Measurement of Stem Cell Proliferation in a 3D Perfusion Bioreactor Using Data-Based Modelling

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The robust and non-invasive monitoring of tissue construct quality inside a bioreactor is a prerequisite for automated construct manufacturing in a clinical setting. The aim of this work is to use a data-based modelling approach to relate the O2 consumption of cells during 3D perfusion culture to their proliferation rate, based on non-invasive microenvironmental O2 concentration measurements. Porous Ti6Al4V scaffolds (510 mm³), seeded with human periosteum-derived stem cells (hPDCs, between 120 000 and 360 000 cells per scaffold), were perfused in a bioreactor capable of measuring O2 concentrations at the in- and outlet of the perfusion chamber. Repeatedly during a perfusion experiment, a 3 hour step in the perfusion flow rate was applied by changing the pump speed from 0.179 ml/min to 0.03 ml/min at normoxic conditions. This specific dynamic flow condition alters the residence time of the medium in the scaffold and consequently the amount of consumed O2 per volume of perfused medium. The dynamic response of the O2 concentration, measured at the perfusion chamber outlet, to the imposed steps in the flow rate can be accurately (R² of 0.995) described by a first order discrete-time, linear single-input single-output transfer function model. Additionally, the model gain parameter shows a strong linear correlation to cell proliferation. This model-based monitoring approach therefore shows potential for non-invasive on-line monitoring of hPDC proliferation in 3D perfusion culture.
Multiphase Modelling of Tissue Growth in a Hollow-Fibre Membrane Bioreactor

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Hollow-fibre membrane bioreactors (HFMBs) are currently under experimental investigation for use in tissue engineering. HFMBs have great potential to control growth of a clinically relevant cell population as the surface area for nutrient delivery is large compared to the bioreactor volume. They consist of a cylindrical glass module with an exit port at either end, through which a synthetic hollow fibre is inserted. Cells are seeded in the extracapillary space surrounding the fibre, and culture medium is pumped into the fibre lumen, reaching the cells via the porous fibre walls which protect them from damage due to high fluid shear stresses. We have developed a mathematical model of a simplified, two-dimensional HFMB, with the aim of understanding how to control cell distribution using flow. We solve for the cell distribution, solute concentration and hydrodynamic water pressure for a number of experimentally-motivated case studies. These setups consider different solutes and flow regimes, and results demonstrate the range of possible behaviours in each scenario. We observe that since solute gradients induce either spatial heterogeneity in cell proliferation rates (in the case of oxygen), or cell movement due to chemotaxis (in the case of a chemoattractant), a careful balance between advection and these effects is required in order to obtain a uniform cell population. In each case we can therefore predict the dependence of cell distribution and yield on prescribed flow rate.
Patient-adapted medicine is one of the most promising approaches to implant larger organ-like structures into a patient and to avoid rejection or inflammatory effects after implantation. We are about setting up an implant to improve the healing process of large bone defects. Therefore, we used scaffold materials doped with human mesenchymal stem cells and introduced an in vitro culture phase prior to implantation. To guide tissue development we mimicked the physiological mechanical conditions similar to the in vivo environment. Since the magnitude of the applied mechanical forces and culture conditions are on a microscopic scale, we applied computational models to predict the culture conditions inside of the scaffold. A variety of fluid simulation results showed the nutrient supply as well as the shear force onto the cells inside of the scaffold in vitro. With this it was possible to develop a perfusion bioreactor solution obtaining a precultured graft for later implant efforts.

One of the physiological stimulus to drive the MSCs into differentiation is the mechanical force they feel in vivo. Therefore, we developed a bioreactor including incubation chamber with linear axle and peristaltic pumps to mimic the physiological conditions the best. Due to the lack of sensors to measure the mechanical force inside of the porous material, we developed in silico studies to calculate the transformed force the cells are exposed to.

We were able to set up studies, showing the upregulation of osteogenic markers (Serpin, SAFB, Fas, ALP, RUNX2, SP7) by using an adequate calcium matrix as well as shear stress trough perfusion in a period of 7 days. Another study shows the impact of mechanical loading relating to gene expression.
Expansion of a Mock Hematopoietic Stem Cell Component in Air-Lift Bioreactor

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Adult hematopoietic stem cells have many applications for curing blood disease as well as blood products. To use the cells in clinical setting, many investigations have recently been done on applying bioreactor for the cell expansion. Air-lift bioreactor is one of the suitable bioreactors for cultivation and growth of cells due to its less shear stress and better distribution of oxygen in comparison with other bioreactors. Also, sampling and the control of conditions are simple in the bioreactor. In this study, proliferation of U937 cell line, as a mock hematopoietic stem cell component, was examined in air-lift bioreactor (volume of 16 mL) with internal loop and sequential batch feeding strategy. The feeding strategy maintained the pH culture in the range of 7.4 - 7.8. Temperature was controlled at 37 °C and 5% CO2 / 95% air with 0.1 vvm was used for aeration through a diffuser in the bioreactor bottom. The results demonstrated cell number increasing to 5.5 fold in the bioreactor and 28.8 fold in T-flask during 8-day culture period. Cell size reduction was also observed in the case of bioreactor, revealing the importance of shear stress in behavior of stem cells in the bioreactor.
A Dynamic 3D Simulation of Tissue-Engineered Construct Growth in Perfusion Bioreactors: an Integrative Approach

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Bioreactors have been utilized in tissue engineering applications in order to provide and maintain a controlled culture environment in terms of mass transport and shear stress distribution. However the time-dependent and interactive relationship between the properties of the cultured tissue engineered (TE) construct and the surrounding bioreactor process environment has not been adequately investigated to date and remains to be deciphered. In this study a 3D coupled model was created capturing porous cell/ECM growth on regular pore scaffolds in a perfusion bioreactor, as well as alterations on the surrounding hydrodynamic environment. Cell/ECM growth was modeled using a level-set function computed over the entire domain, with zero-level corresponding to the cell/ECM-medium interface. Cell/ECM growth was experimentally determined by dynamic culture of human periosteum derived cells on square regular-pore titanium alloy scaffolds in a perfusion bioreactor setup under a flow rate of 1 ml/min. 3D cell/ECM growth rate validation carried out by employing contrast enhanced nanoCT (CE-nanoCT) providing spatial information over a culture period of 21 days. Changes in the hydrodynamic environment caused by cell/ECM growth were further modeled by using the Brinkman equation, providing information on free flow (Stokes equations) and porous medium flow for the cell/ECM fraction (Darcy law). The porosity of the cell/ECM construct was approximated by SEM imaging. The model was subsequently applied to a different regular scaffold geometry predicting accurately 3D cell/ECM growth at different time points for the same flow rate. This computational tool gives new possibilities for scaffold and bioreactor process design for TE applications.
Low frequency pulsed electromagnetic field (PEMF) has proven to be effective in the modulation of bone and cartilage functional responsiveness, but its effect on tendon tissue is still underinvestigated. Since conflicting studies on the effect of PEMF on tendon cells (TCs) have been conducted, we evaluated the possible relation between PEMF dosage and TCs’ response comparing the effects of higher intensity (PEMF-3mT) with single and repeated lower intensity treatments (PEMF-1.5mT and R-PEMF-1.5mT).

TCs, isolated from the waste portion of semitendinosus and gracilis tendons of 7 healthy donors were exposed to different PEMF treatments (intensity: 1.5mT or 3mT; duration: 8 or 12 hours; periodicity: single or 3 treatments with an interval of 48h). DNA content, SCX, COL1A1 and VEGF expression, as well as the release of TNFα and IL10, were assessed at 0 and 2 days after treatment.

All the tested PEMF treatments were able to relevantly enhance cell proliferation, with the exception of 12h R-PEMF-1.5mT, that reduces DNA content 2 day after treatment (-20%; p<.05). All the treatments induced a significant increase of IL6, IL10 and TGFβ release in the culture medium (p<.001), without significantly affecting IL-1β and TNFα production, and thus confirming the feasibility of this approach. 3 mT PEMF reduced the expression of tendon specific markers (SCX, COL1A1), whereas 1.5 mT PEMF, above all as a single exposure, induced their up-regulation as well as the VEGF one. Overall, a 3 mT PEMF intensity seemed to be not beneficial probably because too high, whereas 1.5 mT PEMF, already after the first exposure, allowed to obtain a very good biological response in this in vitro TCs model. Further analyses on different models are needed to confirm these observations.
Exposure to Controlled Equiaxial Mechanical Strain Causes Changes in Proliferation, Pro-Pathologic Programming and Epigenetic Landscape Modifications in Saphenous Vein Progenitors

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Failure of Aorto-Coronary Bypass Grafts (CABG) made with saphenous vein (SV) is a global health problem caused by progressive reduction of grafts patency. This may be related to the modified biomechanical conditions associated to SV arterialization. Progenitors endowed with clonogenic and vascular differentiation ability have been identified in the SV. These cells, named Saphenous Vein Progenitors (SVPs) display potential to differentiate into mesenchymal-derived lineages. The aim of the study was to clarify whether mechanical stress results into SVPs phenotypic changes, so as to justify their involvement in CABG. SVPs were exposed to 10% equiaxial strain for 72h into a dedicated bioreactor (FlexCell). Markers expression, cell growth and epigenetic changes were assessed by cell counting, flow cytometry and western analyses. SVPs cultured under 10% strain underwent a significant reduction of cell growth. Stretched SVPs showed higher levels of myofibroblasts marker Vimentin and enhanced expression of PDGFRβ. By contrast, NG2, β-tubulin (pericyte markers) and, at least in part, αSMA and SM22α (smooth muscle cells markers) were downregulated. Finally, mechanical stretch was associated with a methylation increase in Histone H3 Lysine 36. These results suggest an implication of mechanical strain in pro-inflammatory conversion of human SVPs and indicate a biomechanical cause for inflammatory conversion of SV resident cells in CABG failure.
Magnetic manipulation of hydrogel scaffolds at scales of many hundreds of microns or few millimeters is an emerging, non-contact, versatile, and non-invasive approach for tissue engineering and drug delivery. Recently, assembly of cell encapsulating hydrogels into complex 3D tissue constructs has been performed via utilizing magnetic nano particles (MNPs) and free radicals (FRs). Active magnetic scaffolds have been also developed for on-demand drug and cell delivery by utilizing MNPs. Although release of MNPs from microgels has been presented, release from larger tissue constructs has not been proven for broader tissue engineering applications. Hence, novel approaches are needed to manipulate cell and/or bioactive molecule encapsulating hydrogels without using FRs that decay in a short time or MNPs that are not proven to release from large tissue constructs. Here, we present, for the first time, integration of stable radicals into macroporous hydrogels to form active scaffolds for drug delivery and tissue engineering applications (Figure 1). Our method exploits the paramagnetic properties of stable radicals to assemble in 3D a range of hydrogels including agarose, PEG, and GelMA.

Figure 1: (a) Schematic to manipulate paramagnetic gels. After applying: (b) 15 sec and (c) 1 min magnetic field, magnetic responsiveness of stably radicalized hydrogels for a range of stable radical concentration. Mean ± standard deviation, n = 10 measurements each.
Effect of a Cyclic Uniaxial Strain on Bladder Smooth Muscle Cells and Urothelial Cells Seeded on a Type I Collagen Scaffold

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Several conditions may lead to a dysfunctional bladder, e.g. spina bifida and bladder exstrophy. Currently, bowel tissue is used to augment and restore bladder function but is associated with many complications. There is a need to improve the current treatment modality. Tissue engineering has been recognized as a promising alternative therapy. Cell seeded constructs may provide a good alternative but cell differentiation and alignment needs to be improved. In this study we investigated in vitro differentiation and alignment of porcine smooth muscle cells (SMC) and urothelial cells (UC) under mechanical stimulation and static conditions.

Cells were isolated from fresh porcine bladders, cultured and seeded on a collagen type I scaffold. Mechanical stimulation was achieved in a Bose Electroforce Bio-Dynamic bioreactor, a cyclic uniform uniaxial strain of 20% was applied. Cell proliferation, SEM analysis and, α-SMA, desmin and RCK103 expression of SMC and UC, were determined. RT-PCR was used to examine mechanical stretch induced genes. Immunohistochemistry revealed increased α-SMA and desmin expression in SMC in the stretched constructs. A mechanical stimulus enhanced cell proliferation and SEM analysis showed alignment of SMC. RT-PCR showed increased α-SMA, desmin and calponin expression in SMC cultured under stimulated conditions. No differences were observed in any of the parameters we investigated for UC under dynamic as well as static conditions. Mechanical stimuli induce cell proliferation and differentiation in SMC. Mechanical stimulation of constructs, preseeded with SMC, may improve current tissue engineered bladder constructs with respect to functionality.
Differentiation of pluripotent stem cells into a specific phenotype is a sought-after outcome for advancing bone-related tissue engineering and tissue regeneration applications of these most promising cells. To date, biochemical compounds (e.g., bone morphogenetic proteins) have been utilized to promote differentiation of mesenchymal stem cells (which have the potential to differentiate into osteoblasts, chondrocytes, and adipocytes) into osteoblasts. In contrast, the effects of biophysical stimuli on such outcomes remain unknown. The present \textit{in vitro} study examined the effects of electric current alone (i.e., in the absence of supplemented exogenous growth factors) on the differentiation of mesenchymal stem cells. For this purpose, adult human mesenchymal cells were cultured on flat, indium-tin-oxide-coated glass (pre-coated with fibronectin) in the absence of supplemented exogenous growth factors. A custom-made laboratory set-up was used to expose these cells (passage 3-5) to alternating electric current (10 μA, 10 Hz frequency, sinusoidal waveform), for 6 hours daily for up to 21 consecutive days. Cells exposed to electric current expressed early (TAZ, Runx-2, and osterix), and late (osteopontin and osteocalcin) genes indicative of exclusive osteodifferentiation since genes for the chondrocyte and adipocyte lineages were not expressed.

Osteodifferentiation of mesenchymal stem cells under the conditions examined in the present study can provide critically-needed differentiated cell supplies for tissue engineering and tissue regenerating applications.
Fabrication Parameters of Small Intestinal Submucosa Scaffolds Modulate the Shear-Induced Expression of PECAM-1 and eNOS

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Small intestinal submucosa (SIS) is an extracellular matrix scaffold that has been evaluated as a small diameter vascular graft (<5mm). A large variability in the fabrication parameters used has rendered patency rates of 0-100%. We herein hypothesized that fabrication modifies the microstructure of SIS, which in turn modulates mechanotransduction in endothelial cells (ECs) that populate the scaffold and dictate patency outcome. Four types of scaffolds were fabricated by means of a 2² factorial design that combined: 1) preservation/removal (P/R) of a dense collagen luminal layer, 2) hydration/dehydration (H/D). Structural changes were explored with H&E and SEM. Mechanotransduction was investigated by seeding human umbilical vein ECs (HUVECs) on scaffolds, exposing to pulsatile shear stress (12±4 dyn/cm²) for 1 hour (n=5) in a cone and plate flow system, and evaluating expression of PECAM-1 and eNOS with double-immunofluorescence and qPCR. Differences in the arrangement of collagen fibers were seen between scaffolds, where the dense collagen layer in P grafts formed a lining of the scaffold, as opposed to lose fibers seen in R grafts. H grafts had collagen fibers arranged in bundles whereas D grafts had compacted individual fibers. Expression of both Pecam1 and Enos was significantly upregulated by preservation of the dense collagen layer in scaffolds in a dehydrated state (p<0.1, ANOVA and Tukey’s test). Gene expression in PD/RD was ~32-fold for Pecam1 and ~37-fold for Enos. We conclude that fabrication parameters altered SIS microstructure, and potentially modulated the shear-induced expression of mechanosensitive proteins in HUVECs seeded on these scaffolds.
3D Perfusion Bioreactor Culture of Human Periosteum Derived Stem Cells for Bone Tissue Engineering

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When expanding a progenitor cell population, maintenance of the phenotype is essential. Perfusion bioreactors have been shown to be a valuable tool for cell expansion. However, fluid flow induced shear stress enhances biochemically induced osteogenic differentiation and mineralized matrix deposition. To enable controlled use of bioreactors as culturing vehicle in a tissue engineering process, this study investigated the influence of fluid flow induced shear stress on the proliferation, differentiation and matrix deposition of human periosteal derived cells using a 3D perfusion bioreactor system in the absence of additional osteogenic stimuli. 3D Ti6Al4V scaffolds were seeded with human periosteal derived cells and cultured at three flow rates: static (zero flow rate), 0.04ml/min and 4ml/min, followed by a biochemical and morphological analysis. DNA measurements showed a significant increase in proliferation in the perfusion system as compared to the static control, but no difference was observed between the different flow rates. On the other hand contrast enhanced nanofocus computed tomography indicated that, compared to static culture, there was substantial extracellular matrix formation in the active flow conditions showing a flow rate-dependent morphology. Gene expression analysis showed a slight time dependent increase in proliferation in the perfusion system as compared to the static control, but no difference was observed between the different flow rates. On the other hand contrast enhanced nanofocus computed tomography indicated that, compared to static culture, there was substantial extracellular matrix formation in the active flow conditions showing a flow rate-dependent morphology. Gene expression analysis showed a slight time dependent increase in bone matrix markers. However this increase was significantly lower compared to a static positive control using osteogenic medium. We therefore concluded that, in the absence of additional osteogenic inducers, perfusion alone increases proliferation and extracellular matrix deposition, but does not significantly induce osteogenic differentiation.
Simultaneous Electromechanical Stimulation of ATDPCs for Cardiac Regeneration

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Cardiac tissue engineering aims to develop biological structures capable of reestablishing the physiological organization and function of the myocardium being replaced. It is well-known that cardiac cells are subjected to mechanical and electrical forces, which regulate gene expression and cellular function. Thereby, the challenges associated to mimic biophysical stimuli in vitro are well recognized and have been a major driving force for research in the recent years to improve maturation of therapeutic cells and enhance their electromechanical coupling with native myocardium. We report a novel electromechanical stimulator. The stimulation unit setup was custom made and consisted of a combination of a monophasic programmable electrical device with a custom magnet-driven system. The system enables to stimulate up to 6 culture plates while preserving the sterile barrier. Cells are cultured onto a biocompatible polydimethylsiloxane silicone construct designed to provide structural support to cells, the electrical stimulation electrodes and the magnets. Ultimately, we test the jointly influence of both electrical and mechanical stimulation on adipose tissue-derived progenitor cells (ATDPCs). The parameters tested were synchronized and designed to mimic the physiological heart environment: 2-ms monophasic square-wave pulses of 50 mV/cm at 1 Hz and 10% stretching during 7 days. Preliminary experiments with ATDPCs showed that electromechanical stimulation caused changes in their genetic machinery, increasing important cardiac markers expression. Thus, training of a chosen cell type could direct these cells to a cardiomyocyte-like phenotype and be a valuable tool for further cardiac regeneration purposes.
Biological Influence of Shock Waves Stimulation on Primary Human Tendon Cells (Tcs) Adherent Cultures

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Extracorporeal shock waves (ESW) application has proven to be effective in improving healing and recovery in different musculoskeletal pathologies involving bone and cartilage. Although they are already used as an adjuvant therapy in tendon diseases, the mechanism of their action on this kind of tissue still need to be completely understood. In our study, tendon cells (TCs) were isolated from waste portions of semitendinosus and gracilis tendon of healthy donors undergoing ACL reconstruction. Differently from previous studies in which cells were treated in suspension, we applied ESW to TCs in adhesion, which better resembled the physiologic condition. Cell proliferation and viability were analyzed at 7 and 14 days after treatment, whereas tendon specific markers (SCX, COL1A1, COL3A1) and VEGF gene expression were evaluated at 1, 2, 4 and 7 days after treatment by real time RT-PCR. The release of cytokines (IL-1β, TNFα, IL-6, IL-10, TGFβ), growth factors (VEGF) and metalloproteinases (MMP-3, MMP-13) was assessed by ELISA at the same time points. ESW treatment caused a prompt induction of cells proliferation and viability in comparison to untreated cultures. With the exception of TNFα, the analyzed cytokines and VEGF release was increased by ESW treatment, while MMPs was not affected. Gene expression analysis revealed that SCX transcription was significantly enhanced one day after treatment, while just a slight increase in COL1A1 was observed at day 4 after ESW application. These data support the hypothesis that ESW was able to activate tendon cells, as demonstrated by the increase of cell proliferation, mitochondrial activity, anti-inflammatory cytokines production and lineage specific markers expression.
Electrical stimulation (ES) is a novel modality in the discipline of Tissue Engineering that has been receiving more and more attention in the last few years. This is motivated by the observations that ES can enhance proliferation and differentiation of various musculoskeletal cell lines. Furthermore it is currently used to help the healing of normal and non-union fractures, osteoporosis, osteoarthrosis, lumbar spine fusions and to better integrate implanted biomaterials. Our work focuses on developing electrical stimulation into a novel tool for bone tissue engineering. To this end we developed an autoclaveable capacitive bioreactor system that allows the reliable delivery of pulsed and continuous electric field stimulation up to electrode potential of 500 V. Simulations of the electric field inside the bioreactor were created in the FEM simulation environment COMSOL Multiphysics. Cell culture experiments using commercial bone marrow derived Mesenchymal Stem Cells were performed testing the biocompatibility of the newly developed bioreactor. Further in vitro experiments were carried out assaying the effect of various pulse widths, frequencies and electrode potentials. Their effect was measured upon cell numbers using Picogreen, metabolic activity with Alamar Blue and ECM deposition using Alkaline Phosphatase assays. Our results show that being cultured within the bioreactor without a stimulus has no effect on metabolic activity and only slightly lowers the cell numbers at Day 8. Electrical field stimulation was observed to be able to affect both metabolic and alkaline phosphatase activity significantly at Day 8. We conclude that ES can be a very useful tool for Tissue Engineering.
Mechanical force is an important regulator of bone development. The transduction of mechanical force occurs through mechanosensitive membrane receptors that convert physical force into biochemical signals. Previous work has demonstrated magnetic mechano-activation of key receptors in order to promote the differentiation of stem cells and osteoprogenitors that leads to an enhanced osteogenic phenotype in vitro and in vivo. In this study we demonstrate the use of Magnetic nanoparticles (MNP) to remotely control differentiation of human Mesenchymal Stem Cells (hMSC) and downstream bone development in an ex vivo chick femur model. Femurs from day 11 Dekalb chicks were extracted and organotypically cultured in basal DMEM For 24h. Femurs were then injected with hMSC pre labelled with 250nm magnetite MNP functionalised with either RGD peptide or anti-TREK1 antibodies. Control femurs were injected with functionalised MNP only or with carrier alone. Magnetically stimulated femurs were treated for 10h over 14 days (1h per treatment) in an oscillating vertical magnetic force bioreactor (MICA Biosystems). After 14 days, femurs were fixed in paraformaldehyde and bone density and mineralised volume analysed by micro computerised tomography (microCT). Results showed an increase in mineralised bone volume in hMSC-MNP treated groups compared to control groups by microCT. Further histological analyses indicated new bone was formed around MNP-labelled MSC injected areas in the epiphyseal regions of the femur.

This study demonstrates the potential for remote localised mechanical conditioning in vivo with potential applications for stem cell therapies.
Mechanical stimulation of tissue engineered constructs using bioreactors may prove useful for conditioning them to physiological environments prior to clinical implementation, whilst both mechanical force and biochemical factors play an important role in regulating tissue formation. The aim of this study was to understand the combined effect of cyclic hydrostatic pressure (HP) and growth factor supplemented media on hMSCs seeded in collagen gels. Collagen hydrogels were seeded with hMSCs and cultured for 42 days in either basic unsupplemented media, osteogenic or chondrogenic media (TGFB3). A custom bioreactor (TGT, US) was used to apply 280 kPa cyclic HP, 1hr daily for 42 days. uCT analysis showed increased mineralisation in stimulated samples over unstimulated controls. ALP activity in the media was higher in stimulated samples at all time points. A significant increase in collagen autofluorescence was observed, especially when stimulated gels were cultured in osteochondral media, indicating an increase in collagen remodelling caused by HP. These results suggest that HP and biochemical factors work synergistically to promote osteochondral differentiation and matrix maturation in hMSC seeded collagen hydrogels.
Stem cell therapies (SCTs) offer potential treatment for a vast array of diseases and pathologies. Currently SCTs are delivered through direct implantation, however inaccessible locations, multiple dosages, and patients unfit for surgery, highlight the need for a simpler system of delivery retaining the benefits of direct introduction. Systemic to local delivery using internalised magnetic nanoparticles and external magnetic fields may be the answer. The efficiency of such a technique however may be affected by several circulatory variables.

In this study we detail the effect of haematocrit-induced changes in viscosity and shear on the magnetic trapping of stem cells. Bone marrow and adipose-derived mesenchymal stem cells were internally labelled with super-paramagnetic iron oxide nanoparticles and separately placed in an in vitro circulatory flow model with external permanent magnets. The haematocrit and fluid flow rate were varied and the system run for 1, 3, 6 and 12 hours. Cell trapping was determined using cell counts, and quantification of DNA and protein, along with secondary erythrocyte trapping via assays for haemoglobin and alkaline phosphatase. A viscosity-shear rate profile was calculated for each haematocrit demonstrating blood’s non-Newtonian nature. Our results show that changes in viscosity and shear have a significant effect on the magnitude and time course of the magnetic trapping of both bone marrow and adipose derived stem cells. We also observed a considerable degree of RBC trapping which may have bearing on clinical cell therapies. The results of this study help us to understand the proposed delivery of magnetically labelled cells via the circulation, and the impact of circulatory variables on its efficiency.
Application of a New 3D Concentration Gradient Bioreactor: Building Matrixes with a 3D Gradient of Mechanical Properties

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In this work we show how a new 3D concentration gradient bioreactor could be applied to build hydrogel matrixes with a 3D gradient of mechanical properties. Briefly first we made the mechanical characterization of bulk alginate and polyacrylamide hydrogels, by using different monomer/crosslinkers ratio. The samples were subjected to compression tests to obtain a “map” of hydrogel elastic modulus in function of monomer and crosslinker concentration. Then we used this new bioreactor to impose a three-dimensional gradient of crosslinker to a monomer solution into a chamber, and we let the system to polymerize. The concentration profile was predicted with Computational Fluid Dynamics. Compression tests were made on these matrices, and the formation of an elastic modulus gradient was observed. The important aspect of this research is that we can construct hydrogel matrix with a gradient of mechanical properties, in which cells can be embedded in order to analyze cell behavior as function of substrate mechanical properties. It can have several applications to the study of phenomena related to cell mechanotaxis or differentiation on substrates with different stiffness.
Wound healing requires cell traction forces and matrix re-formation for gap closure. However, how cells remodel their environment depending on the mechanical boundary conditions is to date inadequately understood as is the consequence on macroscopic tissue properties. We hypothesize that cellular contractility and matrix formation are highly influenced by the resistance of the surrounding tissues. To analyse matrix remodelling, we established a novel tissue culture system which allows live monitoring of tissue contraction against mechanical resistance. Macroporous collagen-I scaffolds (1.2±0.4 kPa) were seeded with human dermal fibroblasts. Cell and ECM formation and organization were analysed (actin cytoskeleton, fibronectin, 2nd harmonic generation collagen structuring). Scaffold contraction differed across spring resistances with 5.0% at 6.57N/m and 12.3% at 1.42N/m while free floating samples led to 26.9% contraction. Final contraction force was 1.9 times higher in the stiffer spring wire (2.01mN±0.4) compared to the softer one (1.04mN±0.1). Surprisingly, cells showed a three-phase force/contraction behaviour: (1) delay phase with moderate increase, (2) pronounced cell force build-up phase and (3) saturation of contraction phase. Cell behaviour changed from phase 1 to 2 from a direct cell micro-environment interaction (organization within a scaffold pore) to a coordinated contraction of the cells beyond the scale of single cells. The later phase led to distinct collagen I network orientations – scaffolds switched from isotropy to anisotropy over time but dependent on mechanical resistance. The results highlight the role of macroscopic boundary conditions in cell organization and cell-scaffold interaction.
Development Of Novel 3D Uniaxial Strain Device to Model Cancer Cell Metastasis

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As tumors enlarge and progress, tumor cells are exposed to a myriad of altered mechanical forces that could dramatically modify their behavior. The objective of this study is to create a three-dimensional (3D) in-vitro model for understanding how mechanical strain affects the morphology and migratory potential of collagen encapsulated prostate cancer (PC-3) cells. A novel mechanical loading platform was developed to apply 1% uniaxial strain to 3D collagen encapsulated PC-3 constructs. In this study, strain was applied for 30 minutes daily for 3 days. The experimental groups included a control (unloaded) group, a cyclic loaded group (1Hz frequency), and a static loaded group (continuous strain application). At day 3, samples were analyzed for cell elongation, actin polymerization, and migratory potential. Static loading significantly increases cell elongation, as compared to control and cyclic loading conditions (p<0.001). Increased cell elongation is indicative of the morphological change associated with EMT. Additionally, actin polymerization analysis shows static loading to significantly increase actin polymerization, compared to control and cyclic loading (p<0.001). This suggests possible increased migratory potential. Spatial actin analysis shows greater peripheral actin accumulation in static loaded constructs. This, in conjunction with the increased migratory tendencies seen in static loaded samples, suggests static uniaxial stretch increases migratory tendencies of prostate cancer (PC-3) cells.
Mesenchymal stem cells (MSCs) are widely implicated as a cell source for tissue engineering of skeletal tissue in cell based therapy. Mechanical forces can stimulate the differentiation of MSCs in micro-environment and the resulting mechanotransduction would provide crucial adjuncts to customary biochemical signalling pathways. Combining microfluidic systems with mechanical stimulation for osteogenesis represents scientific and technological innovation that would greatly impact the regenerative medicine. We demonstrate a microfluidic chamber design for mechanical stimulation of flexible cellular microspheres and possible a high-throughput microfluidic system for parallel processing of stem cell aggregation. We also showed that collagen microspheres can act as an efficient cell supporting system, with proper viability and migration efficiency post encapsulation. A microfluidic chamber was made of PDMS consisting of a main channel of 1.5mm with a central smaller channel of 0.7mm for compression of the collagen beads. Movement of the microsphere and media is obtained using syringe pumps. 2.5µl Collagen microsphere containing Passage 3 heMSC with collagen concentration of 0.5 and 1mg/ml and cell density of 1025 cells per droplet were made. Cell viability and behaviour were monitored up to day 9 post capsulation.
Complexity of neural cells requires several factors during designing materials to mimic natural niche. A material aimed to be used for culturing neural cells should contain bioactive epitopes to make it as similar to extracellular matrix (ECM) of nervous tissue. Short peptides derived from cell surface receptor activating parts of ECM proteins are commonly used to incorporate bioactivity to synthetic scaffolds. Bioactive scaffolds can induce adhesion, migration, proliferation or even differentiation depending on the epitopes included. Electrical conductivity is another important property of neural cell scaffolds, since neurons respond to electrical stimulation. In order to develop a multifunctional scaffold supporting neural cell survival and providing signals for differentiation, we exploited conductive peptide nanofibers. PA nanofibers were used to carry bioactive epitopes derived from laminin as well as having glycosaminoglycan mimicking moieties to enhance growth factor signalling. Electrical stimulation of PC-12 cells on these bioactive and conductive peptide nanofiber substrates induced cells to extend much longer neurites in addition to upregulation of neural marker genes.
A new in situ strategy for heart valve regeneration has been proposed, less time-consuming and with reduced infection risks as compared with the traditional in vitro approach. In order to investigate the role of physiological loads on in situ tissue formation and remodeling, the effects of deformation on matrix production and on release of chemokines, capable to trigger a positive inflammation-mediated response, will be assessed. Human peripheral blood mononuclear cells (PBMCs), freshly isolated from healthy donors, were seeded onto polycaprolactone-based strips and subjected to 5% and 10% cyclic strain at 0.8 Hz by a FlexCell® tension system. PBMCs and human myofibroblasts (HVSCs) seeded onto non-loaded scaffolds served as control. After 1, 2, 4 and 7 days of straining, strips were sacrificed and DAPI staining was performed to assess cell distribution throughout the scaffolds. After 7 days of straining, the amount of cells and glycosaminoglycans (sGAGs) was evaluated by biochemical assays. DAPI staining showed that PBMCs were uniformly distributed throughout the scaffolds. Straining had no effect on sGAGs production and proliferation of PBMCs. Overall sGAGs production was lower for PBMCs compared to HVSCs. Future analyses will involve cell distribution and chemokines gene expression assessment after 1 and 2 days, chemokines and matrix genes expression and immunohistochemistry after 4 days. Tissue formation after 7 days will be further assessed by histology and quantification of collagen amount.

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The development of a successful bioartificial kidney (BAK) faces some obstacles such as overgrowth and dedifferentiation of epithelial cells. It is known that some renal epithelial cells are exposed to hypertonic environment in nature and it modulates the synthesis of extracellular matrix (ECM) proteins, which is known to influence the performance of cells. Therefore we aim to investigate whether hypertonic treatment could improve the performance of epithelial cells. Cells were treated with regular (300 mOsm) and hypertonic media (400 and 500 mOsm) and were investigated by MTS, qPCR and microscopy. We found that hypertonic media suppressed the mitochondrial activity, suggesting the suppression of overgrowth of cells. Evaluation of morphology, immunostaining pattern and mRNA level of epithelial and mesenchymal markers revealed that hypertonic media maintained an intact and differentiated epithelial monolayer, while isotonic medium did not. mRNA expression of ECM genes was not influenced, suggesting that hypertonic effects were not due to modulation of ECM genes. This prompted us to explore other underlying molecular mechanisms such as tonicity-responsive enhancer binding protein (TonEBP), which is the key transcription factor that protects the cells from hypertonic deleterious effects. To fulfill this, we silenced the mRNA expression of TonEBP and it appeared that it played a major role in the observed hypertonic effects. Our results are promising for BAK, since they suggest that hypertonicity inhibits the overgrowth of the cells and maintains an intact differentiated epithelial monolayer, which are some of the major bottlenecks in the development of BAK. In the future we aim to validate these effects in vivo.

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The capacity to control cell fate using scaffolds specifically designed is one of the major challenges of Tissue Engineering field. Nowadays construction of 3D artificial substrates is not simple and it is possible to modify their properties changing material and chemical composition as well as morphological and physical properties. In this study scaffolds with similar morphology but different chemical and physical properties were produced. In particular, a synthetic polymer (Poly D, L-Lactic Acid) and a natural polymer (silk-fibroin) were used to fabricate solvent casting particulate leaching scaffolds which were morphologically and mechanically characterized. Human osteosarcoma derived osteoblasts (MG63) or Human Mesenchymal Stem Cells (hMSC) were seeded onto the scaffolds using an osteogenic medium to investigate biological response and tissue formation. The results show the beneficial influence of the natural derived silk-fibroin on cell proliferation and collagen production and assembling. Moreover, it was evident the control exercised by the mechanical properties on the final osteoblast calcium deposition. This work is indeed significant because it was possible to trigger and prove different osteoblast responses conducive to bone-tissue formation.
A Method to Control the Mechanical Stretching of 3D Cell-Laden Hydrogels

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The cell microenvironment is a complex three dimensional (3D) matrix providing cells with a number of biochemical, biomechanical and bioelectrical signals that control cellular processes. Among others, mechanical forces influence the organization of the extracellular matrix (ECM) as well as the cellular behavior, contributing to define tissue functional properties. Thus, the recapitulation of the natural 3D mechanical field experienced by cells is a crucial aspect in the development of physiologically relevant \textit{in vitro} tissue models. To this aim we implemented a procedure for mechanically stimulating cells embedded in 3D hydrogel matrices. Photopolymerized hydrogels (Gelatin Methacrylated, GelMA) embedding vascular primary cells isolated from porcine aorta were mechanically stimulated (1-10\% strain at 1 Hz) exploiting an actuator-driven stretching bioreactor. Cell-laden hydrogels were bonded to polydimethilsiloxane (PDMS) sheets to allow the transmission of the stimuli to cells. A reliable adhesion between hydrogels and substrates was achieved through the functionalization of PDMS with benzophenone followed by UV irradiation. Preliminary results on cell morphology and viability demonstrated the biocompatibility of both the hydrogel photopolymerization and the substrate functionalization processes. A cellular alignment along the stretching direction was also detected through fluorescence analysis in the mechanically stimulated samples compared to the static controls. Thus, by tuning hydrogel composition or mechanical stimulation parameters, the proposed procedure can be applied to \textit{in vitro} investigations of cell mechanics, allowing for independent regulation of stiffness and strain.
Effect of Joint Mimicking Load on the Zonal Organization of Engineered Cartilage

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Articular cartilage is highly organized tissue distinguished by cell arrangement and macromolecular distribution. Mechanical forces are known to develop the zonal organization of adult cartilage, unlike juvenile cartilage with even distribution of cells and extracellular matrix. We hypothesized that engineered cartilage -- which has a limitation in histological appearance unlike its native counterpart -- can be reorganized by mechanical stimulation. In this study, we constructed a joint mimicking loading system and examined the effect of the stimuli on engineered cartilage. Chondrocytes were obtained from polydactyl patients and embedded at density of 5 x10⁷/ml in Fibrin/HA gel. The constructs were placed under the femoral condyle-shaped device of the loading system designed to produce shear and compression forces and were stimulated for 1 hour per day. The control group was static-cultured without any stimuli. After 4 weeks, the cell arrangement and macromolecular (sGAG, Collagen 2, and Lubricin) expression were analyzed through histology; the mechanical properties were also measured. In the stimuli group, cells were reorganized horizontally and vertically to the surface on the superficial and deep zones of tissue, respectively. Lubricin was expressed in the surface of the stimuli group, but not in the control group. Larger amounts of GAG and Collagen 2 were generally expressed on the stimuli group but peripherally expressed on the control group. The mechanical property of stimulated tissue was higher than that of the control group by twofold. Our loading system could generate mechanical stimuli similar to those in the joint and successfully reorganize engineered cartilage to make it analogous to native cartilage.
Modeling the Magnetic Force-Controlled Colonisation of Fibrous Scaffolds by Stem Cells

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To engineer functional tissues, cells must often be controllably seeded into porous scaffolds. This may involve selective retention of subpopulations of target cells from a suspension, e.g. when using rare stem cells. One option is to incubate the cell suspension with antibody-conjugated magnetite beads. Subsequently, a magnetic field is applied, which attracts them through the culture medium into the scaffold. Because of many independent variables involved, the optimization of this procedure is difficult. Here we present a mathematical model that describes the collection of magnetic particles-labeled cells and scaffold colonization. The model incorporates magnetic, viscous drag and buoyancy forces, and is parameterized against experimental data on the cell and bead sizes, number of conjugated beads per cell, the magnetic force and scaffold porosity (Figure 1). Model simulations predict particle trajectories and scaffold densities for varying distributions of conjugated beads per cell and of collection times. Finally, the impact of varying the magnetic field and scaffold porosity is explored within the model, with a view to developing experimentally robust strategies that generate a spatially uniform distribution of cells throughout the scaffold.

Figure 1 The experimental setup. A 300 um thick fibrous scaffold obtained by electrospinning from poly-caprolactone is set into the base of a petri dish. 1ml of tissue culture medium is pipetted into the dish, and a second fluid layer containing the cell suspension is added on top. The cell suspension contains a given number of target cells presenting an antigen, with varying numbers of magnetic beads attached. The dish is placed on a magnet for various intervals (5-20 min).
Arrayed Stimuli-responsive rGO\textsubscript{SH}/PMA\textsubscript{SH} Microcapsule on Conductive Substrate for Neural Tissue Engineering

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In this study, a novel material to encapsulate nerve growth factor (NGF) into graphene and polymer capsules for applications as tissue engineering was developed by firstly functionalizing thiol group onto the surface of rGO nanosheets for the self-assembly rGO\textsubscript{SH}/PMA\textsubscript{SH} LbL onto the mesoporous silica microcapsules. The well-ordered rGO\textsubscript{SH}/PMA\textsubscript{SH} particle layer with microscale topography was arrayed on the flexible ITO substrate and used for an extracellular matrix to accelerate the PC12 cell proliferation and differentiation by controlling release of a nerve growth factor (NGF). Under electric stimulation, the rGO\textsubscript{SH}/PMA\textsubscript{SH} microcapsule stimulated the NGF releasing and accelerated the proliferation and differentiation of the PC12 cells. The average neurite lengths could enhance quadruple after culture on the rGO\textsubscript{SH}/PMA\textsubscript{SH} microcapsule substrate for one day compared to those without coating rGO\textsubscript{SH}/PMA\textsubscript{SH}. The results suggested that the surface topography of arrayed rGO\textsubscript{SH}/PMA\textsubscript{SH} microcapsule, NGF chemical cue and electrical stimulation increase the cell activities. This result demonstrates that the rGO\textsubscript{SH}/PMA\textsubscript{SH} microcapsules may be used as potential substrate for neural regeneration and neural prosthetics in tissue engineering applications.
Dynamic Cell Culture: Improving Culture of Bladder Smooth Muscle Cells on Collagen-Based Constructs

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Several conditions such as bladder exstrophy and neuropathic bladders can cause dysfunction of the urinary bladder. Augmentation cystoplasty is a common treatment to restore urinary bladder capacity and function. However, this treatment is associated with several complications, including metabolic abnormalities, infections, stone and tumor formation. The use of tissue engineered constructs may reduce these side effects. This study focused on combining primary bladder smooth muscle cells with type I collagen constructs and culture under static and dynamic conditions, aiming for new regenerative urological therapies. Flat constructs were prepared from highly purified bovine type I collagen. Primary rabbit smooth muscle cells were seeded overnight in a rotator and subsequently cultured for 6 days in static and dynamic conditions. Constructs were analyzed by scanning electron microscopy, histology and immunofluorescent staining. The constructs showed a highly porous network. Static and dynamic cultures were compared for differences in cellular distribution and differentiation status. Dynamic culture resulted in cellular alignment, desmin positive cells and a higher cell density and distribution compared to static culture.

In this study we show that it is possible to increase cell density and distribution by dynamic culture of type I collagen constructs. Cellular alignment and differentiation was observed in dynamic culture, but not in static culture. Optimization of the stimulation protocol may result in more effective culture conditions to create fully conditioned tissue engineered constructs for new treatment options in urological surgery.
Topology Optimization of Porous Scaffold Architectures Incorporating Culture Conditions for Tissue Engineering

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Mechanical stimuli play an important role in the development of functional scaffolds for tissue engineering due to its effect on cell proliferation and cell differentiation. Computational approaches that suggest how to analyze the relationship between critical parameters such as scaffold porosity, Young’s modulus and dissolution rate using mechanoregulation algorithms exist. The mechanical and fluid flow responses of scaffolds have also been coupled within synthesis frameworks that allow for the design of scaffolds with optimum stiffness and permeability from scratch. This paper presents a synthesis framework based on the integration of these two concepts to improve the design of porous scaffolds further. More specifically, a topology optimization framework based on analysis models that account for cell differentiation and degradation of the scaffolds using existing mechanoregulation algorithms and in-vivo experiments as well as mechanical and fluid flow analysis is proposed. Mechanical analysis module is coupled with fluid flow analysis and integrated to an additional PDE module in COMSOL Multiphysics to represent scaffold degradation. All equations are solved simultaneously and integrated within a level-set based topology optimization framework. In-vivo experiments based on stem-cell based artificial scaffolds that were produced using a bio-plotter are used for validation. Computational results show that the optimal topology meets desired mechanical and fluid-flow properties. Comparison of cell differentiation and degradation response via in-vivo experiments and the mechanoregulation response of the designed scaffold show that the design strategy is promising in designing functional porous scaffolds for tissue engineering. Initial results show that coupling of mechanical, fluid-flow and cell response yields scaffolds that can be designed from scratch to meet desired tissue functionalities using the proposed synthesis framework. Incorporation of experimental data is currently underway for further model improvement. Provide scaffolds with improved properties and for the tissues that are in interest. Stiff, permeable enough, well-degradable scaffolds will provide better housing for stem cells. The analyses aforementioned will be examined and improved with their fabrication.

Keywords: Computational scaffold design, degradation, COMSOL
Effects of Electrical Stimulation and Substrate Topography on the Cardiomyogenic Commitment of Human Progenitor Cells

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We present a biomimetic in vitro cell culture platform combining electrical stimulation (ES) with topographical cues to promote cardiac commitment of human progenitor cells (hPCs) from adipose tissue and cardiac muscle. The multi-chamber ES culture system consists of: i) 12 independent Petri-like culture chambers made of polydimethylsiloxane (PDMS) with two embedded stainless steel electrodes propagating the ES within the cell-seeded surface, ii) a custom-made electric stimulator generating programmable electrical pulse stimuli, iii) a I/O hardware data acquisition system, for real-time monitoring of the ES pattern. Microgrooved cell substrates (parallel grooves of 25 μm width and 5 μm depth) were obtained through spin-coating of PDMS on a silicon wafer mold fabricated through photolithography process. hPCs were seeded on collagen-coated smooth and grooved substrates, cultured for 24h in static condition, and then exposed to monophasic electric pulses (5V, 2ms, 1Hz) for further 24h. Substrates’ topography enhanced cell elongation and alignment, while ES induced the up-regulation of the expression of connexin-43 (Cx43) mRNA, as observed by preliminary qRT-PCR experiments. The immunofluorescence analysis for Cx43 revealed the protein translocation at the cell membrane, suggesting that ES promoted the maturation of functional cell-cell electro-mechanical coupling (gap-junctions). Further experiments are ongoing to evaluate the expression of genes related to the cardiac lineage commitment. The developed cell culture platform, allowing the delivery of multiple and highly controlled physical stimuli, represents an effective tool for standardized and high-throughput analysis of stem cells conditioning.
Vocal Fold Augmentation with Injectable Polycaprolactone Spheres and Theromosensitive Pluronic F127 Hydrogel: Long-term in vivo Study for the Treatment of Glottal Insufficiency

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Glottal insufficiency due to incomplete contact between the two vocal folds may be caused by several glottal conditions such as vocal fold palsy or paresis, presbylaryngis, scarring, and sulcus vocalis. There is increasing demand for reconstruction of glottal insufficiency. Several injection materials have been examined for this purpose, but all had limitations, such as poor long-term durability, migration from the injection site, inflammation, granuloma formation, and interference with vocal fold vibration due to viscoelastic mismatch. Here, we developed a novel injection material, consisting of polycaprolactone (PCL) microspheres, which exhibits better viscoelasticity than conventional materials, and Pluronic F127 carrier, which decreases the migration of the injection materials. The material was injected into rabbits with glottal insufficiency and compared with the FDA-approved injection material, calcium hydroxylapatite (CaHA). Endoscopic and histological examinations indicated that PCL/Pluronic F127 remained at the injection site with no inflammatory response or granuloma formation, whereas CaHA leaked out and migrated from the injection site. Therefore, vocal fold augmentation was almost completely retained during the 12-month follow-up period in this study. Moreover, induced phonation and high-speed recording of vocal fold vibration showed decreased vocal fold gap area in the PCL/Pluronic F127 group. Our newly developed injection material, PCL/Pluronic F127, permits efficient augmentation of paralyzed vocal fold without complications, a concept that can be applied clinically, as demonstrated by the successful long-term follow-up.
Immunomodulatory Nanoparticles from Elastin-Like Recombinamers

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Elastin-like recombinant polymers (ELRs), a new subclass of protein-based recombinant polymers, are composed of the pentapeptide VPGXG, which mimics the hydrophobic domain of elastin. Their lack of immunogenicity along with their biodegradability and biocompatibility for human tissue, tissue fluids, and blood, make these polymers exceptional candidates as carriers in vaccine-delivery approaches. Likewise, the modular nature of ELRs allows different amphiphilic elastin-like block corecombinamers (ELbcRs), which are able to self-assemble with high reproducibility and homogeneity in a thermally driven process in an aqueous medium, to be constructed. We developed a new ELbcR-based vaccine carrier that self-assembles into highly monodisperse and stable nanovesicles that can be used to present low antigenic peptides, e.g., from M. tuberculosis. The biosynthesis of ELbcR allows the production of large quantities using an efficient and cost-effective inverse transition cycling (ITC) purification procedure. The compositional complexity of vesicles is retained after secondary processes such as endotoxin removal, sterilization, and lyophilization. The diameter (60 nm) and transition temperature (15°C) of these vesicles favor a long half-life of the presented antigen in circulation, thus allowing sufficient time for uptake and processing. In vivo immune-challenge experiments in a mouse model point to the induction of a biphasic response characterized by the early secretion of chemotactic cytokines (innate-immunity phase), followed by the induction of a Th-2 immune response for the Ag-ELbcR vehicle, as shown by the late onset of IL-5 production and the up-regulation of IgM and IgG antibody responses (adaptive-immunity phase).
In-vivo Brain Target Imaging and Protein Delivery Using Functionalized Pluronic-Based Nanogel

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Blood-brain barrier (BBB) acts as a protective barrier for the central nervous system (CNS) due to tight junctions and lack of fenestration, and thereby blocking the delivery of protein drugs or therapeutic agents to the brain. Herein, our work demonstrated the successful delivery of β-galactosidase to mouse brain overcoming the BBB by using functionalized Pluronic-based nanogel. A specific peptide target ligand and chitosan were used to functionalize the Pluronic-based nanogel, and an NIR-dye (cy5.5) was also conjugated to the nanogel for in-vivo imaging. Then, β-galactosidase was loaded into the nanogel as a model protein drug to the brain. The in-vivo brain accumulation of the nanogel was monitored after intravenous injection. Both the peptide and chitosan were necessary for successful targeting of the nanogel to the brain, and β-galactosidase was also delivered efficiently accordingly.
Liquified Capsules Combining Immunoprotection and Microcarriers

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Bioencapsulation is an evolving branch of tissue engineering (TE), providing a wide range of therapeutic treatments, such as for diabetes, bone and cartilage defects. In this study we focused on the three essential characteristics that dictate the functionality of bioencapsulation systems, namely, permeability, mechanical stability, and biocompatibility. Our aim was to address a main issue in a TE perspective related to the fact that liquified environments enhance the diffusion efficiency but most cells are anchorage-dependent, thus need to adhere to a solid structure.

We propose a new concept that combines the excellent diffusion of liquified environments with solid microarchitectures to provide cell adhesion sites. As a proof-of-concept, capsules composed by a multilayered membrane of poly(L-lysine) (PLL), alginate, and chitosan encapsulating modified poly(L-lactic acid) microparticles were developed. The build-up was monitored by QCM-D analysis. Thickness measurements using the Voigt viscoelastic model and mechanical assays were performed. Results show that PLL modulated the type of regime growth, resulting in a more resistant and thicker film. The ability of capsules to support cell survival was assessed. In vitro assays, such as MTS, live-dead, DAPI-phalloidin, and DNA quantification were performed. Results were compared with hydrogel particles and empty capsules. Capsules with microparticles revealed an improved biological outcome with higher cell metabolic activity and proliferation.

We believe that our strategy brings a real breakthrough in the bioencapsulation field, allowing to tailor different properties in one structure and to use combinatorial approaches, such as drug delivery and co-culture systems.
Electrochemically synthesized Ag/ poly(N-vinyl-2-pyrrolidone) hydrogel nanocomposites for biomedical applications

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Nanocrystalline silver is often used as a component of nanocomposite materials, due to its antimicrobial properties and healing activity. Silver nanoparticles (AgNPs) are being applied into different healthcare products such as burn dressings, scaffolds, skin donor and recipient sites, drug delivery applications. Stabilization and better dispersion of AgNPs in aqueous media is achieved by hydrogels, acting simultaneously as nanoreactors for in situ synthesis of AgNPs while providing easy handling. An innovative method was developed for production of AgNPs incorporated in poly(N-vinyl-2-pyrrolidone), PVP, hydrogel (Ag/PVP nanocomposites), based on the electrochemical reduction of Ag⁺ ions within the swollen PVP hydrogel, which was performed in situ, under constant applied voltage. Ag/PVP nanocomposites were evaluated by investigation of the sorption characteristics in simulated body fluid, silver release kinetics, mechanical properties under bioreactor conditions simulating in vivo conditions in the articular cartilage and antimicrobial activity. The optimal experimental conditions were determined by varying the applied voltage and implementation time, using UV-visible spectroscopy. All results suggested that Ag/PVP nanocomposites meet the requirements needed for wound dressing application. The sterility of Ag/PVP nanocomposites is preserved even after 28 days of silver release, since about 20 % of the initial silver content still remains inside nanocomposite.
The Addition of Cobalt-Bioactive Glass Microparticles into Collagen-Glycosaminoglycan Scaffolds to Enhance Osteogenesis and Angiogenesis

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A significant problem with tissue engineering large tissue constructs is achieving functionally vascularised constructs that can integrate with the host tissue. Attention is thus directed towards the delivery of pro-angiogenic factors to facilitate angiogenesis. The avenue explored herein is the activation of the cellular Hypoxia Inducible Factor (HIF-1α) pathway which responds to hypoxic conditions by activating pro-regenerative genes such as Vascular Endothelial Growth Factor (VEGF). In this study, the HIF-1α pathway was targeted using cobalt, a known hypoxia mimic, conjugated to osteogenic bioactive glass (4CoBG) for controlled ion release. The combination of 4CoBG within a collagen glycosaminoglycan (CG) scaffold optimised for bone repair may elicit an enhanced angiogenic response. To test this we first analysed the effect of 4CoBG (38µm or 100 µm diameter) incorporation on CG scaffold micro architecture and bioactivity. BG improved the mechanical properties of the composite scaffolds which remained highly porous (<98%) relative to the CG control. Cobalt was released in physiologically relevant concentrations for HIF-1α activation (3-15ppm), which upregulated VEGF gene expression and led to increased accumulation of VEGF protein from endothelial cells cultured in direct contact with the scaffolds. Moreover, the presence of BG served to induce differentiation of preosteoblasts as evidenced by enhanced alkaline phosphatase, cell mediated calcium production, as well as enhanced matrix mineralization. In conclusion, a ‘smart’ scaffold with dual osteoinductive and angiogenic potential has been successfully developed and may be of benefit in achieving vascularisation of constructs designed for orthopaedic applications coupling osteogenesis with angiogenesis.
Impact of Surface Modifications of Nanoparticles on Uptake and Subcellular Localization in Human Mesenchymal Stem Cells
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Major applications for nanoparticles in regenerative medicine include labeling, tracking and manipulation of mesenchymal stem cells. We analyzed the uptake and intracellular localization of silica nanoparticles in human bone marrow-derived mesenchymal stem cells (MSC) in dependence on chemical modifications of the surface. Particles with a size of 100 nm were functionalized with hydroxyl groups (OH), amino groups (NH₂) or carboxyl groups (COOH). Cell viability was not affected when using a particle concentration up to 500 µg/ml. Using flow cytometry, we observed a maximal uptake of nanoparticles within two hours. Uptake of NH₂-nanoparticles by MSC was lower compared to OH and COOH modified particles. Using confocal laser scanning microscopy to visualize various organelles, the subcellular localization of the particles was studied. Live cell imaging revealed a colocalization of NH₂-particles with mitochondria, whereas COOH particles were localized in lysosomes. Both cell proliferation and osteogenic differentiation were not affected by particle uptake. To see, whether a chemical modification of the particle surface is suitable to use nanoparticles for transfection of DNA and express genes, we found that surface modification of nanoferrit particles (BNF-starch) by poly-D-lysine is a promising approach. Using these modified particles GFP was expressed in MSC with a highly effective rate.

In conclusion we demonstrated that chemical modification of the surface is crucial for quantitative uptake and intracellular fate of the particles as well as for gene transfection.
Enhanced Efficacy of Magnetic Nanoparticles Against Antibiotic Resistant Biofilms In The Presence of Metabolites

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The emergence of methicillin-resistant Staphylococcus aureus (MRSA) is a major cause of hospital-acquired infections (HAI). There is an urgent clinical need to develop new strategies to reduce infection, without resorting to antibiotics for which bacteria are developing a resistance towards. In this study, we designed superparamagnetic iron-oxide nanoparticles (SPION) to treat MRSA biofilms and showed that SPION efficacy increases with metal conjugations in the presence of fructose metabolites. SPION were synthesized using a high temperature synthesis method and were conjugated with 1 mM or 4 mM salts of ZnCl₂ and FeCl₃. A 6.70 log reduction was observed when MRSA biofilms were treated with unconjugated SPION (UC) (p < 0.001). Metal conjugations (Fe1, Fe4, Zn1, Zn4) increased the SPION efficacy (p < 0.001). Moreover, conjugated SPION were significantly more effective than vancomycin (p < 0.05). For the first time, it was shown that the anti-biofilm efficacy of UC (p < 0.05) as well as 1 mM Fe³⁺ (Fe1) (p < 0.05) and 1 mM Zn²⁺ (Zn1) conjugated SPION (p < 0.001) was significantly increased in the presence of fructose. We have shown that SPION are more effective than currently used antibiotics for treating MRSA biofilms (especially, vancomycin). For the first time, metabolic stimulation is shown to enhance the efficacy of magnetic nanoparticles. It is envisioned that this simple and inexpensive approach could lead to novel alternative treatments to the only clinical option, vancomycin.
Investigation of IgG Adsorption Behavior on Spherical Albumin Biosorbents in Simulated Body Fluid

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The blood plasma is one of the basic material for the diagnosis of diseases. Removal of abundant proteins will help in the discovery and detection of less abundant proteins that may prove to be informative disease markers like Alzheimer disease. The importance of analyzing bioactivity in-vitro prior to in-vivo analysis is quite clear. Invivo studies require animal sacrifices, are more costly and less easily reproducible, and involve ethic issues. SBF is an aprotic and acellular solution containing different salts that simulate the concentration and pH of human plasma. In this study we preferred protein based (albumin) biosorbent because it is non-toxic, biocompatible and can be prepared at desired properties. Albumin biosorbents were prepared as microsphere form by emulsion polymerization method. 0.1g/ml albumin concentration, 1000 rpm stirring rate, 1% glutaraldehyde concentration 30 minutes crosslinking time were determined as optimal conditions. Prepared albumin microspheres had mean diameters 10±2 µm at these conditions. Adsorption of IgG onto biosorbents were performed at batch wise in normal simulated body fluid (SBF) at room temperature. Albumin biosorbents thus obtained were treated with IgG (initial conc. 8.5 mg/ml). Albumin capturing capacity was 291.6≈292 mg IgG/g polymer (% 85.8 ≈86) in SBF. There was no leakage at washing with water. We reached up to 85.8 % IgG removal amount and it may be concluded that albumin biosorbents are sufficient in terms of efficiency of IgG removal. Albumin biosorbents have better removal capacity when compared with the other polimeric biosorbents. We believe that albumin biosorbents offered the promising approach with good removal specificity and efficiency of IgG.
Biodegradable and biocompatible nanoparticles have important applications in medicine as drug carrier, diagnosis, therapeutic and imaging applications. Chitosan nanoparticles widely used in medical applications due to their unique properties such as biocompatibility, biodegradability, low toxicity and bioadhesivity. Synthesis methods and conditions of nanoparticles affect their physical and chemical properties. Therefore, the aim of the present study was to optimize the preparation of alginate coated chitosan nanoparticles and determine their loading capacity. Chitosan nanoparticles were prepared ionic gelation method based on the interaction between the negative groups of the pentasodium tripolyphosphate and the positively charged amino groups of chitosan. Various concentrations of chitosan and TPP solutions were used at two different pH value (4.5-4.7). First, chitosan nanoparticle were loaded with bovine serum albumin and then loaded chitosan nanoparticle suspensions were added dropwisely into sodium alginate solution. The suspension was centrifuged to obtain nanoparticles. Finally, alginate coated chitosan nanoparticles were formed by crosslinking of calcium chloride (CaCl₂) aqueous solution which presents on the surface of chitosan nanoparticle. The size and zeta potential of the nanoparticles were analyzed by dynamic light scattering (Zetasizer), morphological analysis were determined using atomic force microscopy (AFM). The loading capacity and releasing efficiency of nanoparticles were characterized and examined by UV spectrophotometry.
Efficacy and Safety of Nanohybrids Comprising Silver Nanoparticles and Silicate Clay For Controlling \textit{Salmonella} Infection

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Developing effective and safe drugs is urgent for replacing antibiotics and controlling multidrug-resistant microbes. Nanoscale silicate platelet (NSP) and its nanohybrids, silver nanoparticle/NSP (AgNP/NSP), have been developed and the nanohybrids show a strong and general antibacterial activity \textit{in vitro}. Here, their efficacy for protecting the \textit{Salmonella} infected chicks from fatality and septicemia was evaluated. Both orally-administrated NSP and AgNP/NSP, but not AgNPs alone, effectively reduced the systemic \textit{Salmonella} infection and the mortality. In addition, the quantitative silver analyses demonstrated that the silver deposition from AgNP/NSP in the intestines was less than that from the conventional AgNPs, indicating that the presence of NSP for immobilizing AgNPs reduces the Ag accumulation in the tissues and improves the safety of AgNPs. These \textit{in vivo} evidences illustrate that both NSP and AgNP/NSP nanohybrid represent potential agents for controlling enteric bacterial infections.
Iron oxide based magnetic nanoparticles have extensive potential applications in the field of regenerative medicine. The magnetic behavior of the particles can be tuned by modifying the iron oxide core to better suit the end application. Transition elements such as zinc and cobalt have been shown to alter the magnetic properties of iron oxide nanoparticles upon doping, but may also affect the biocompatibility of the material. In this study biogenic cobalt doped magnetite nanoparticles, produced by iron-reducing bacteria, have been studied to assess the toxicity imparted upon doping cobalt into a magnetite core. Citric acid coated biogenic samples with different levels of cobalt doping (Co$_x$Fe$_{3-x}$O$_4$; x=0.13, 0.38, 0.42, 0.71, 1) were compared to pure magnetite (Fe$_3$O$_4$) and CoCl$_2$, the latter a known cytotoxic salt of cobalt. Cytotoxicity was assessed in MG63 and bone marrow derived primary mesenchymal stem cells by quantifying live/dead staining via spectrofluorimetry and flow cytometry. A concentration range of 10 to 500μM was assessed and cells incubated with the additives for 3 days. Magnetite-exposed cells showed only a modest loss of viability with increasing concentration while for CoCl$_2$ cell viability rapidly decreased. The higher Co-doped magnetite samples tested, showed loss in viability at higher concentrations but viability did not fall as rapidly in comparison to CoCl$_2$. This study sheds light on the leeway available to dope and hence modify magnetic behavior, whilst not compromising on biocompatibility when designing novel magnetic nanomaterials.
Magnetic "Cyborg" Cells for Tissue Engineering

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Here we report a facile approach for direct one-step magnetic functionalization of viable human cells with cationic polymer-stabilized magnetic nanoparticles (MNPs) and the use of MNPs-functionalized "cyborg" cells in fabrication of two-layered tissue prototypes. We employed A 549 epytheliocytes and human skin fibroblasts to produce three-dimensional porous lung tissue-mimicking multicellular clusters. The MNPs-coated cells were viable and proliferated normally, as determined using viability stains, MTT-test and Cell Index measurements in real time. The clusters had the distinct porous alveoli-mimicking morphology, to a certain extent reconstituting the real lung tissue structure. We believe that the use of surface-functionalized magnetic cells is advantageous because of the low toxicity and high control over the magnetization.
PLGA Porous Scaffolds Containing Magnesium Nanoparticles Prepared Using Ice Microparticles as a Porogen for the Suppression of Inflammation

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Porous biodegradable scaffolds fabricated with aliphatic polyesters, such as poly(lactic acid) (PLA) and poly(lactic-co-glycolic acid) (PLGA), still have many problems, although have been extensively utilized for tissue engineering. Such scaffolds frequently show inflammatory response at the early stage after implantation into the body. Polyesters undergo hydrolytic degradation via the bulk erosion mechanism by the random scission of the ester backbone. It degrades to acidic monomers, a normal human metabolic by-product. However, inflammatory response may be severe under the condition in which the acidic product accumulates and is concentrated. In this study, it is demonstrated that the addition of magnesium nanoparticles (MgNPs) can suppress inflammatory responses in PLGA porous scaffold by using ice microparticles as a porogen material. Ice microparticles were prepared by spraying cold deionized water into liquid nitrogen. PLGA (75:25, 110K) was dissolved in methylene chloride and the solution was mixed with MgNPs. Then, ice microparticles were added to a pre-cooled PLGA solution. Finally, the mixture was frozen by being placed in mold in liquid nitrogen and then freeze-dried to form porous scaffold. For the suppression of inflammation, PLGA scaffolds incorporated with MgNPs were fabricated with different ratios of the MgNPs. The prepared MgNPs were characterized by FT-IR, TGA, and DLS. The morphology of the PLGA scaffold was observed by SEM and the PLGA scaffolds have a well-defined porous structure in the presence of MgNPs. The quantitative analysis of MgNPs was evaluated by TGA and ICP. In addition, cell culture using the scaffold will be discussed.
Antimicrobial Effect of Allicin, Silver Nanoparticles and their Combination Against Skin Infection due to *Pseudomonas aeruginosa* in Animal Model

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Common antibiotic may be useless against multiple pathogens because of the multidrug resistacy. Therefore new ways of nanotechnology and effective combination of various antimicrobial mechanisms can be the solution for this problem. The aim of this study was to investigate the antimicrobial effects of allicin, silver nanoparticles and their combination against skin infection due to *Pseudomonas aeruginosa* in animal model. Based on the macrodilution susceptibility test, the minimum inhibitory concentration (MIC) and Minimum Bactericidal concentration (MBC) of allicine, silver nanoparticles and their combination were investigated. Skin infection was produced in the shoulder region of 12 mice using *Pseudomonas aeruginosa* and the effect of ointment of allicin, silver nanoparticles and their combination were evaluated. The results showed that, MIC and MBC of allicin for *P. aeruginosa* were 2.38 and 4.77 mg/ml, respectively. MIC and MBC of silver nanoparticle for *P. aeruginosa* were 3.12 and 6.25 ppm, respectively. MIC and MBC of combination of allicin and silver nanoparticles on *P. aeruginosa* were 0.59 mg/ml and 1.5 ppm, 1.19 mg/ml and 3.12 ppm, respectively. Anti microbial effect of allicin, silver nanoparticles and the synergistic activity of their combination against skin infections due to *Pseudomonas aeruginosa* was observed in mouse model. The results showed that allicin in combination with silver nanoparticles have synergistic effect against skin infection due to *Pseudomonas aeruginosa*.
Use of microbes in production of nanoparticles has received increasing attention due to growing need to develop environmentally benign technologies as novel approach. They can be produced in various size and shape (circular, cylindrical etc.) by controlling synthesis conditions. Many microorganisms produce inorganic materials either intra- or extracellularly. Some of Gram positive and Gram negative bacteria are able to adsorb and take up metal ions and capable of selectively reducing certain metal ions. Microbiological method forms nanoparticles by cheap and green process however the time required is the major drawback of this method. A novel combinatorial synthesis approach by using a combination of culture supernatant of bacteria and microwave irradiation is extremely rapid, simple and green. In this study, production of nanoparticles was carried out using the *Bacillus* strain isolated from rhizosphere of *Acacia cyanophylla* Lindley. To eliminate the time constraint, microwave irradiation were also employed. The possibility of manipulating the size and shape of silver and gold nanoparticles was investigated. Differently sized and shaped nanoparticles were successfully synthesized. These remarkable results gave us an opportunity to employ these nanoparticles in a number of applications from electronics to biology, pharmaceutical and medical diagnosis and therapy.
“Cell-Like” Compartmentalized Vehicles with Temperature and Magnetic Responsiveness

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Cells are multifunctional structures which, due to a unique hierarchical and compartmentalized organization, allow numerous compounds to be transported, proteins to be synthesized and enzymatic reactions to be regulated within their cytoplasm. Inspired by the cells’ structure, we present compartmentalized gel beads with temperature and magnetic-based responsiveness, and hierarchical organization ranging from the nano to the visible scales. Liquefied alginate macroscopic beads coated with a layer-by-layer chitosan/alginate shell served as containers both for model fluorophores and microcapsules, which in their turn encapsulated either another fluorophore or magnetic nanoparticles (MNPs). The microcapsules were coated with a chitosan/elastin-like recombinamer (ELR) shell, the latter being a genetically engineered polypeptide exhibiting temperature responsiveness. By varying the temperature from 25 °C to 37 °C, the 2-hour release of rhodamine encapsulated within the microcapsules and its diffusion through the external compartment decreased from 84% and 71%. The devices could withstand handling and centrifugal stress, with 50% remaining intact at a rotation speed of 2000g. MNPs attributed magnetic responsiveness towards external magnetic fields, as a biomimetic approach inspired by the unique properties of magnetotactic bacteria.

Such customizable system can be envisaged for targeted delivery and localized immobilization allied with sustained drug delivery at physiological temperature and protection from premature drug leakage. The mild conditions used can also envisage the use of these systems as multifunctional reservoirs to transport bioactive agents and cells in tissue engineering applications.
Polymeric Microparticles for Cell Isolation and Injectable Cell Carriers for Tissue Engineering

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Recently, we showed that chitosan films modified with specific biochemical cues could be used as a method for cell separation. In addition, published works suggested the use of chitosan microparticles as cell carriers and at the same time building units of new scaffolding systems to be used in tissue engineering (TE). This suggested that chitosan microparticles could be effectively used to isolate target cell populations, work as cell microcarriers for cell expansion and potentially as an injectable system to form well characterized constructs for TE. The isolation of specific cell phenotypes could be of special interest, as within stem cells there are many subpopulations with different capabilities to differentiate through different lineages and thus with distinct therapeutic potential. In this study chitosan microparticles were functionalized with specific antibodies to isolate target cells. The functionalization process starts by the immobilization of biotin in the particles, followed by incubation with streptavidin and in a later step, incubation with a biotinylated antibody. Immunofluorescence was performed to assess the immobilization efficiency, results reveal successful attachment of the molecules. In vitro tests reveal that the particles were able to select specific cell types from mixed cell populations, maintaining cell viability. To test the capacity of the particles as an injectable and instructive scaffold, seeded particles (with targeted isolated cells) were injected into a PDMS mould and incubated up to 4 weeks. In vitro results demonstrated the capability of the microparticles as an injectable scaffold that support cell growth. The method here presented can provide platforms for automatic selective cell isolation while also be amenable as a well characterized injectable system.
Chitosan (CHI) is a biopolymer that has been explored for biomedical applications mainly due to its biocompatible and biodegradable properties. Unless it has been modified, CHI is soluble in acidic media which turn it incompatible with cells or fragile molecules encapsulation. Superhydrophobic surfaces (SHS) appeared as a very simple, fast and cheap technology to produce spherical hydrogel particles, involved by a dry environment (without aggressive surrounded media, pressure, stirring and high/low temperatures) providing mild conditions to encapsulate, with very high efficiency, cells and/or bioactive agents. In this work, using SHS, we produced particles using CHI (without any chemical modification) to encapsulate cells or drugs. A CHI solution was firstly neutralized to reach pH 6.2 with a weak base (β-glycerophosphate, β-GP); afterwards L929 cells or a model drug (dexamethasone) were dispersed in such CHI formulation. The suspension was then dispensed onto polystyrene SHS with controlled volumes acquiring a spherical shape. Finally, tripolyphosphate (TPP) was added on the top of each sphere allowing the ionic crosslinking of the CHI at RT. The possibility to tailor the pH by using β-GP allowed obtaining a system capable to maintain the cells viable. Moreover, the crosslinking provided by TPP was weak and the β-GP increased the elastic modulus of this kind of gels when subjected to physiological temperature. This characteristic may be interesting if it is desired to implant the particles by minimal invasive procedures, where the particles are in a soft state during the implantation, and harden inside the body. The pH responsive properties of CHI particles allow to obtain distinct drug release profiles under different pH dependent mediums.
Dendrimer Nanoparticles Diffuse in the Brain Parenchyma Following Subarachnoid Administration

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Nanoparticles are promising tools for CNS regenerative medicine, namely as drug delivery carriers that can diffuse in the nervous tissue and release therapeutics in a controlled manner. However, the brain barriers considerably limit molecule entrance and mobility into the CNS. Moreover, even slight differences in the chemical composition of these molecules can drastically affect its trafficking in the CNS. In the present study we administered FITC-labeled methylprednisolone-loaded CMCh/PAMAM dendrimer nanoparticles (NP) in the cerebrospinal fluid (CSF) of healthy Wistar rats in order to investigate its distribution in the brain tissue 72 hours post-administration. We chose to use an uncommon yet less invasive and equally effective administration technique in the cisterna magna. Electron micrographs of the NP revealed a spherical morphology of around 109 nm in diameter, also confirmed by zetasizer measurements. The drug release was quantified in vitro by HPLC and a sustained release was detected during 14 days. Confocal imaging of cryo-sectioned preparations revealed the presence of NP throughout the brain parenchyma, predominantly in the prefrontal cortex, hippocampus and lateral ventriculus. Moreover, no morphological or expression alterations were observed in astrocytes following GFAP staining, indicating that the NP do not negatively affect glial cell functions.

Figure 1 – A. Intracisternal method of administration. B. Prefrontal cortex preparation stained for GFAP (red, astrocytes) revealing the presence of FITC-labeled methylprednisolone-loaded CMCh/PAMAM dendrimer nanoparticles (green). C. Higher magnification showing astrocytes (red) and internalized NP (green).
Enhanced Wound Healing Effect of Canine Adipose-Derived Mesenchymal Stem Cells with Low-Level Laser Therapy in Athymic Mice

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Adipose-derived mesenchymal stem cells (ASCs) are attractive cell source for skin tissue engineering. However, one obstacle to this approach is that the transplanted ASC population can decline rapidly in the recipient tissue. The aim of this study was to investigate the effects of low-level laser therapy (LLLT) on transplanted canine ASCs in a skin wound animal model. LLLT, ASC transplantation (ASCs) and ASC transplantation with LLLT (ASCs + LLLT) were applied to the wound bed in athymic mice. Wound healing was assessed by gross evaluation and by hematoxylin and eosin staining. The survival, differentiation and secretion of vascular endothelial growth factor and basic fibroblast growth factor of the ASCs were evaluated by immunohistochemistry and western blotting. The ASCs and ASCs + LLLT groups stimulated wound closure and histological skin regeneration. The ASCs + LLLT group enhanced the wound healing, including neovascularization and regeneration of skin appendages, compared with the ASCs group. The ASCs contributed skin regeneration via differentiation and secretion of growth factors. In the ASCs + LLLT group, the survival of ASCs was increased by the decreased apoptosis of ASCs in the wound bed. The secretion of growth factors was stimulated in the ASCs + LLLT group compared with the ASCs group. These data suggest that LLLT is an effective biostimulator of ASCs in wound healing that enhances the survival of ASCs and stimulates the secretion of growth factors in the wound bed.
As the importance of the lymphatic vascular system is increasingly recognized, it is now attractive to bio-engineer both, human blood and lymphatic vessels in one tissue or organ graft. For the first time, we show here that the simultaneous bio-engineering of lumen-forming and branching lymphatic and blood capillaries and their integration into dermo-epidermal skin grafts is possible. The generation of human lymphatic capillaries within 3-D hydrogels strictly required the presence of primary dermal fibroblasts. Lymphatic lineage specific markers, such as Prox1, Lyve-1 and Podoplanin, allowed the unequivocal distinction of lymphatic and blood capillaries both in vitro and in vivo. According to their lymphatic nature, the engineered capillaries responded to both lymphangiogenic and anti-lymphangiogenic stimuli, thus demonstrating the value of this system also for assaying lymphatic vessel formation in vitro. Importantly, neither blood and lymphatic endothelial cells intermixed during vessel development, nor did blood and lymphatic capillaries anastomose under the described circumstances. Taken together, we are now able to assemble human keratinocytes, fibroblasts and endothelial cells into functional dermo-epidermal substitutes that contain the two synergizing blood and lymphatic vascular plexuses. It is to be hoped that as further insights into the regulation of safety, quality and ethical standards will occur, the described skin grafts can be soon translated into a true benefit for patients suffering from severe skin defects.
Knocking-out Smad3 Creates a Benefit Niche for Allogenic Mouse Fetal Skin Development in Adult Wounds

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Stem cell mediated tissue regeneration needs an optimal environment for stem cell function. Fetal skin development represents a process of the interaction between skin progenitor cells and their unique extracellular matrix niche, which is also important for the mechanism study of skin progenitor cell differentiation and fetal scarless wound healing. Additionally, the change of niche environment such as altered expression levels of growth factors or cytokines may also change the outcome of fetal skin development. This study tested the hypothesis that the deletion of Smad3 creates a wound environment favorable for fetal skin development. Green fluoresce protein (GFP) fetal mouse skin (C57/B6) of gestational day 16.5 days was transplanted to the wound beds of wide-type (WT), heterozygous (HT) and homologous (KO) Smad3 deletion mice (C57BL/6×129SV) respectively. The results demonstrated that GFP fetal skin after its transplantation developed much better into hair follicle contained skin in KO wound beds than in HT and WT wound beds with significant differences in follicle numbers among three groups at 1, 2 and 3 weeks post-transplantation (p<0.05). In addition, much less fibrosis was observed in KO wounds than in HT and WT wounds with significant difference in wound bed thickness among three groups at 2 and 3 weeks post-transplantation (p<0.05). Interestingly, there was delayed graft rejection in KO group when compared to HT and WT groups. In conclusion, deletion of Smad3 in a wound bed creates a better environment favored for skin progenitor cell differentiation and fetal skin development. Translation of such a concept to the creation of a wound environment favored for adult stem cell differentiation and skin appendage formation may become an important strategy for the regeneration of wounded skin.
Histological Analysis of Transplanted Autologous Epidermal Cell Sheets in a Porcine Model

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Endoscopic submucosal dissection (ESD) is an effective treatment for early esophageal carcinoma. However, as a complication, en bloc large resection of esophageal mucosa, especially circumferential ESD, induces severe stricture. We succeed in preventing severe esophageal constriction after circumferential ESD by transplanting fabricated autologous oral mucosal epithelial cell sheets in human clinical settings. In the present study, we confirmed the survival of autologous epidermal cell sheets (AECSs) after the endoscopic transplantation onto artificial ulcer after circumferential ESD, and chronologically examined engraftment of AECSs in a porcine model. Epidermal cells were isolated from porcine skin tissue, and cultured on temperature-responsive cell culture inserts for 2 weeks. After circumferential esophageal ESD, AECSs were endoscopically transplanted to artificial ulcer. Macroscopic and histological examinations were performed after 1- and 2-week after the transplantation. Cytokeratin (CK) 1 and CK14-positive cells were presented in AECSs, whereas expression of CK1 was disappeared. In post-transplantation 1 week, fluorescently-labeled AECSs were detected on the surface of the ulcer. Moreover, in immunohistological analysis, CK14 and CK17 were expressed, although expression of CK1 was disappeared. In post-operation 2 weeks, expression level of CK14 was maintained, but CK17 positive cells decreased. Transplanted AECSs remained for at least 1 week after grafting. In addition, it was suggested that transplanted AECSs altered the pattern of keratin expressions in accordance with surrounding environment.
Immunomodulation of Human Inflammatory M1 Macrophages by Collagen I Matrices Containing Artificially Sulfated Hyaluronan

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Unopposed activation of inflammatory macrophages (M1) is assumed as major cause for persistent inflammation in chronic wounds and aberrant implant healing. Thus biomaterials capable to modulate macrophage activation and to promote inflammatory resolution represent a promising approach for treatment of non healing wounds and to promote implant integration into functional tissue. Since native ECM is known to guide functions of immune cells we tested artificially compounded ECM (aECM) on their capability to modulate functions of inflammatory M1. Artificial ECM were composed of collagen I and hyaluronan (HA) which was additionally modified by introduction of sulfate groups resulting in low- and high-sulfated HA derivatives, respectively. Testing macrophage functions revealed reduced inflammatory activities of M1 differentiated on collagen I matrices containing high-sulfated HA (hsHA). Release of TNF and IL-12 by these M1 are significantly reduced due to impaired activation of NFκB. Moreover, these macrophages are capable to secrete immunoregulatory IL-10 typically not produced by M1. Cytokine ratio of IL-12/IL-10 created by macrophages on this aECM is similar to that produced by regulatory M2 macrophages. Since these M1 also show reduced activity of transcription factors STAT1 and IRF5 both controlling macrophage polarization to M1-subsets we conclude that matrices composed of collagen I and hsHA dampen inflammatory macrophage function by impeding signaling pathways crucial for polarization and activation of inflammatory M1. We therefore suggest this aECM as promising biomaterial for modulating inflammatory macrophage functions during implant healing and in chronic wounds.
Biodegradable Membranes for Human Bioartificial Skin Substitutes

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Reconstruction of bioartificial skin substitutes is a major issue to restore functional and esthetical integrity in burn patients and to develop in vitro models for toxicological test alternatively to animal experimentation. Human bioartificial skin substitutes as in vitro models allows a rapid, accurate and highly specific evaluation of the potential toxicity and effects by the topical exposure of a wide range of chemical, cosmetic, pharmaceutical and medical products. Polymeric membrane acts as support for cell adhesion, oxygenation and carbon dioxide removal favouring cell growth and proliferation. An additional challenge in the realization of skin substitutes is represented by the use of biodegradable materials that allow the maintenance and the proliferation of cells resorbing in a controlled fashion. In this study biocompatible and biodegradable polymeric membranes were developed and tested for the realization of human bioartificial dermal and epidermal substitutes by culturing human fibroblasts and keratinocytes on biodegradable membranes of chitosan (CHT), polycaprolactone (PCL) and a biosynthetic blend of CHT-PCL synthesized by phase inversion process. Biodegradability, morphological and physico-chemical membrane properties were characterized and the cell adhesion, proliferation and morphology were evaluated on the different substrates. The morphological and physico-chemical membrane properties influenced in particular the differentiation of human keratinocytes towards a complete proliferative bioartificial epidermal substitute or to a specific epidermal strata. These bioengineered constructs provide an interesting approach for manipulating in vitro skin tissue reconstruction providing a valid tool for therapeutic and drug-screening applications.
Combining Cell Sheet Technology and Human Adipose Stem Cells for the Treatment of Full-thickness Wounds


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Tissue Engineering remains as the most promising approach to target Skin Regeneration problematic upon major loss or trauma. At the forefront, different and sophisticated approaches using stem cells have been proposed. Human adipose stem cells (hASCs) have gained special attention due to their facilitated accessibility and isolation, their immunosuppressive features, but particularly due to the secretion of factors known to be relevant to restore healthy skin tissue. This work proposes the use of cell sheet (CS) engineering to fabricate 3D constructs of hASCs, taking advantage of their deposited natural extracellular matrix (ECM), the cohesive cell-cell interactions and respective molecular milieu, to promote mice full-thickness skin wound regeneration. CS of hASCs were obtained from both thermoresponsive (TR) and standard culture surfaces, respectively by temperature reduction and mechanical peeling. Independently of the strategy used to obtain the CS, human cells were found at the wound site 21 days post-transplantation. Although no transdifferentiation was detected, our findings showed that transplanted hASCs affected neotissue vascularization. Additionally, an extended effect on epidermal morphogenesis, evidenced by the presence of rete ridges–like structures, and a significant number of hair follicles, was observed in the group treated with CS obtained from the TR surfaces. The considerably higher stability of the constructs and the natural adhesive nature of the respective CS obtained by temperature decrease seem to significantly contribute to the obtained results by potentiating the interaction between the transplanted and resident cells thus influencing the different aspects of skin regeneration.

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Proteomic Analysis of Skin Graft Taking

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Introduction: The goal of this study was to identify protein expression during skin graft taking in order to apply this knowledge to skin tissue engineering. Materials & Methods: The modified dorsal skinfold chamber was performed in B6 mice (n=30). Autologous full-thickness skin grafts were transplanted and harvested for proteome analysis after 0, 1, 3, 5 and 10 days. Each mouse underwent intravital microscopy to characterise the stadium of graft take and vascularisation (0d=normal skin, 1d=no perfusion, minor phenotypic angiogenic capillary changes, 3d=graft reperfusion, 5d= graft angiogenic response with bud formation, 10d= reestablishment of normal skin capillary pattern, no angiogenic changes). Subsequently the protein fraction was separated in a 2D approach (3gels/timepoint), followed by MS and MS-MS protein identification. Results: 52 differentially expressed proteins and their expression pattern were identified. A number of proteins could be assigned to the NO pathway. Arginase-1 was found to be decreased leading to an increase of active eNOS. Complementary to this, Ca\(^{2+}\) binding proteins (Sorcin, Parvalbumin, Tetranectin, Troponin T) showed a decreased expression, leading to increased level of Ca\(^{2+}\) ions required for eNOS activation. Other identified candidates (HSPB1, HSP6) belong to the group of heat-shock proteins, which are known to be involved in cell migration. Conclusion: The proteomic approach proved its suitability to deliver new insights into the process of skin graft taking on the protein level. Further analysis is on-going revealing the involvement of novel proteins in engraftment. This knowledge may be beneficial for tissue engineering of skin in the future.
A Novel Compromised Full Thickness Wound Model Using a Partially Excised Burn for Evaluation of dDermal Substitutes


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The assessment, and thus development, of new synthetic skin replacements is hampered by a lack of suitable pre-clinical models. In porcine full thickness excision the response is vigorous, in contrast to the most challenging clinical situations which could gain most benefit from treatment. Surgical excision of burn wounds normally includes the ischaemic penumbra to create a ‘bleeding wound bed’, but experimental excision leaving the ischemic zone intact may establish a delayed healing response. We aimed to characterize the healing trajectory of incompletely excised full thickness porcine experimental burn wounds, and determine the usefulness of this model for evaluating synthetic dermal scaffolds (using Smart Matrix, Matriderm) & split thickness skin grafts. All wounds were dressed & wound healing (measurements, photography, Eykona scanning, laser Doppler and histological analysis (8mm punch biopsies) was observed at weekly intervals for 42 days. Compared to acute full thickness wounds, partially excised burn wounds show delayed contraction and inflammatory granulation, and progressively increased microperfusion to 3 weeks. Histology showed persistent wound bed oedema resolving over 6 weeks, and a markedly delayed inflammatory granulation, infiltrating the hypodermis slowly over 3 weeks, without the definitive superficial fibrosis of the acute healing response. Dermal scaffolds accelerated healing, but the inflammation score varied markedly between the different scaffolds. This partial excision burn wound model creates a marked and reproducible delay and compromised healing profile with a notably attenuated granulation response. The potential for assessing different wound healing therapies is shown.
Pre-existing Capillaries Guide Regeneration of Bio-engineered Dermo-Epidermal Skin Grafts

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Despite considerable progress in bioengineering prevascularized dermo-epidermal skin grafts (PDESG), little is known about the effects of prevascularization on the regeneration of these grafts after transplantation. We bioengineered fibrin hydrogel-based PDESG as well as non-prevascularized DESG (npDESG) employing keratinocytes, fibroblasts and endothelial cells isolated from human skin. Using defined co-cultures consisting of dermal fibroblasts and human dermal microvascular endothelial cells, we generated a high density of mature, pericyte-covered microvessels and analyzed the effects of prevascularization on DESG take and regeneration in vivo. We show that the pre-existing microvessels rapidly connect to the recipient’s vasculature after transplantation and accelerate the ingrowth of capillaries from the wound bed. Furthermore, prevascularization significantly improved the regeneration of DESG by increasing the survival of co-transplanted cells, accelerating the remodelling of fibrin into collagen-1 and shortening the inflammatory response. Notably, prevascularization was found to induce the formation of human skin-like rete ridges. Our results underline the significant advantage of a pre-existing capillary plexus in bioengineered dermo-epidermal skin grafts prior to their transplantation.
Extracellular Calcium Enhances the Wound Repair Capacity of Dermal Fibroblasts

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The direct application of calcium to skin wounds through calcium alginate dressings has been shown to be beneficial. The effect of these dressings on cellular events is mediated by the release of calcium into the wound bed, however it is currently not known how Ca²⁺ released from these dressings influences cells in situ. In this work we have created an in vitro environment with different calcium concentrations and investigated the response of rat dermal fibroblasts to it. Proliferation of dermal fibroblasts was assessed in the presence of low Ca²⁺ concentrations (1-4 mM). The chemotactic activity of extracellular calcium was determined in a Boyden chamber system. Dermal fibroblasts repair studies were performed by in vitro wound closure assays. A collagen assay was used to detect collagen production of fibroblasts. The expression of the calcium sensing receptor (CaSR) on dermal fibroblasts was visualized by immunostaining. An increased proliferation was found when culturing with 2 and 2.5 mM Ca²⁺. This effect was abolished when antibodies that blocked CaSR were used. The migration of dermal fibroblasts was increased in cells treated with 2 mM Ca²⁺. Furthermore, increasing extracellular Ca²⁺ accelerated dermal fibroblast repair. Higher amounts of collagen were observed as the extracellular Ca²⁺ concentration increased. The CaSR was found to be expressed in fibroblasts cultured with extracellular calcium.

This work has shown that low Ca²⁺ concentrations influence the proliferation, migration, repair, and collagen synthesis of dermal fibroblasts. A better understanding of how dermal fibroblasts respond to calcium may help to develop novel regenerative therapeutics allowing the regeneration of a functional epidermis in skin disorders.
Incorporation of Olive Leaf Extract into Silk Fibroin/Hyaluronic Acid Sponge Matrices for Wound Dressing Application

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Wound dressing provides therapeutic and protective features and promotes natural healing process when applied to a wound area. Being non toxic and immunologically inert, natural biopolymers have potential in fabrication of wound dressings. Growth factors, antimicrobial and antioxidant agents can also be used in functionalization. In this study, olive leaf extract incorporated silk fibroin (SF) and hyaluronic acid (HA) sponge matrices were prepared. SF is a well known biopolymer for its biodegradability, biocompatibility and low inflammation risk. HA, as a natural component of extracellular matrix, was used to promote cell migration and proliferation. Olive leaf extract has a combination of antimicrobial and antioxidant effect due to its composition of phenolic compounds. Clearing pathogenic microorganisms and scavenging against increased amount of reactive oxygen species in the wound area, it has high potential in wound healing. In order to investigate difference in morphology, degradation and release properties, process parameters were changed. HA addition in soluble form promoted pore structure. Pore formation and robustness of the material changed in parallel with HA amount, affecting degradation and release properties. Olive leaf extract showed low cytotoxic effect on fibroblast cells and high antioxidant activity. Extract also formed clear zones compared to antibiotics against *Escherichia coli* and *Staphylococcus epidermidis*. Wound healing properties of soluble extract and cell attachment on extract loaded materials were also investigated in terms of biocompatibility. Both soluble and loaded form of extract promoted cell migration and attachment.
Wound contraction is an essential component of skin wound healing. Excessive contraction that causes scarring and fibrosis is undesired. Wound contractures compromise healing, lead to disfiguration, and limit function. Although several approaches have been introduced to improve wound healing, the need for novel effective means to reduce undesired wound contraction remains.

In this study we investigated the effect of nanofibrillar cellulose hydrogel on wound contraction in comparison with a clinically used cellulose-alginate gel (Purilon®). Porcine models of full thickness wounds (8mm biopsy wounds and 4x4cm wounds covered with a 1:3-meshed split thickness skin graft) were used. Wound contraction was observed macroscopically, and histological sections of the wounds were made at the end of the 14-day follow-up. Nanofibrillar cellulose hydrogel inhibited 80% of biopsy wound contraction, whereas cellulose alginate had no effect. However, application of nanocellulose on split thickness skin grafts did not inhibit epithelialization of the interstices or cell migration from the graft. Our results, although preliminary, indicate a potential for nanofibrillar cellulose hydrogel as a novel material for inhibiting wound contraction and preventing scarring.
The Production of Bio-engineered Skin Grafts under Good Manufacturing Practice

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The two skin substitutes denovoDerm and denovoSkin, developed at the Tissue Biology Research Unit in Zurich, Switzerland, are to be tested in phase I clinical trials in the context of the FP7 funded EuroSkinGraft consortium in Zurich. In European Union and in Switzerland, the regulations for Advanced Therapy Medicinal Products (ATMPs) have changed markedly with the introduction of the regulation EC 1394/2007. After a transitional phase until end 2011, it now is obligatory for all ATMPs to comply with this regulation. For clinical trials, it has become mandatory to produce ATMPs under strict GMP conditions. Producing autologous skin grafts in a GMP facility is extremely demanding and poses a significant challenge for research teams that works in an academic setting. Prominent obstacles to overcome are the costs, which include the rent for a GMP facility, additional personnel and detailed in-process controls. For a production process that was previously perfectly established under experimental conditions, changes have to be made concerning GMP grade (or medical grade) starting materials, consumables and protocols. This means in most cases a time- and money consuming process of adaptation and testing. However, after all we are confident that we will succeed in coping with the “GMP-challenge” in collaboration with the regulatory authorities and for the sake and safety of our patients.
In vitro and In vivo Performance of Bioabsorbable Scaffolds Suitable for Skin Regeneration

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Temporary scaffolds for tissue engineering require bioabsorbable materials that degrade into non-toxic substances once the tissue is repaired. Since polyhydroxyalkanoates satisfy these properties, many researchers have focused on them to build up bioabsorbable scaffolds to be harvested with osteoblasts, fibroblasts, vascular cells, among others. Drawbacks of these biopolymesters are their hydrophobic behaviour and the slow bioabsorption rate, which affect negatively the formation of the new tissue. Furthermore, these devices must have suitable mechanical properties to prevent friability, adapt to anatomical contours, facilitate handling by surgeons and, last but not least, a proper surface to enhance the adhesion, motility and proliferation of cells and the interaction with the surrounding tissue.

This work shows that proper fabrication processes may allow getting a hydrophilic scaffold with proper biodegradation rate and suitable biomechanical properties for skin regeneration. On one hand, the decrease of the dry weight of samples stored at 37°C and pH 7.4 in phosphate buffered saline (PBS) and PBS with lipases as well as changes in the molecular weight of PHBV experimental show how the degradation rate depends on the mesostructures and the media.

On the other hand, the scaffolds were implanted into excisional wounds on the abdomen of adult Wistar rats to test biological activity in a wound healing model. In some rats the scaffolds accelerated wound healing relative to the blank control; in all cases almost complete healing with reepithelization was achieved at day 10. H&E-stained sections show the absence of inflammatory response, the complete regeneration of the skin and a partial bioabsorption of the scaffold 10 days after implantation.
Preliminary Study for the Effect of PDGF or Mesenchymal Stem Cells on Tissue Repair of Cutaneous Radiation Injury

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Purpose: Osteoradionecrosis (ORN) of the mandible is a serious complication of radiation therapy, and preceded by soft tissue damage before bone loss appears. However, there is still no adequate treatment to heal the soft tissue damage of ORN. This study investigated the injection time-dependent effect of PDGF-BB or rat mesenchymal stem cells (rMSCs) on radiation-induced soft tissue injury. Rat model was designed to irradiate the skin of SD rats while sparing the body and internal organs by utilizing a non-occlusive skin clamp along with an x-ray image guided stereotactic irradiator. All wounds were created using the 50 Gy dose level both on the right and the left flank at a 100 cm source-to-surface distance. Next day, experimental groups were randomly divided into three groups (n=3-4, each group). Left side in a subject was administered by 8 µg PDGF-BB, rMSCs or the combination of PDGF and rMSCs, while the right side was used as vehicle control. Cytokine or rMSCs was injected immediately (Group 1) or on 14 days after irradiation (Group 2) showing maximal ulceration. The healing effect was analyzed by defining the percentage of the irradiated area ulcerated in each wound at given time points, and histological observation. No systemic or lethal sequelae occurred in any animals, and all irradiated skin areas in the multi-dose trial underwent ulceration. Greater than 60% of skin within each irradiated zone underwent ulceration within 16 days. Experimental groups were all reached peak ulceration above 50%, with all healing significantly but incompletely by the 56-day endpoint compared with control group. PDGF showed better efficiency in the wound repair while there was little difference in Group 1. PDGF-BB treatment groups (only PDGF group or PDGF and rMSCs mixed group) improved healing quality more highly organized collagen fiber deposition in full-thickness compared with control group. These results suggest that PDGF-BB or MSCs are an alternative as a treatment to heal soft tissue injury, highlighting future therapeutic options, particularly for patients suffering from an impaired capacity for ORN. Injection point was also a critical factor in wound healing, and more effective at the time having peak ulceration after irradiation, rather than immediate injection.
The Biological Properties Difference by Preservation Methods of Allografts

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Skin allografts are considered the common management of severe burns injury. The allografts provide mechanical and physiologic barrier wounds from microbial contamination, provide artificial bilayers instead of inborn skin, promote re-epithelialisation and pain decrease by wound site. The purpose of this research was to analyze the biological properties of some allografts skin by preservation method (Fresh skin, glycerol preservation, cryopreservation). The clinical outcomes into patients treated with various allografts were compared and it was observed that viable allograft cells may play a role in reducing the risk of progression to SIRS. We analyzed the difference on preservation techniques and biological properties, as well as tissue viability, immunogenicity and antimicrobial activity by the preservation methods. Through the mechanism of these allografts is still nuclear but these data should support a prospective and clinical study.
A Novel In vitro Model of 3D Cell Ingress into Scaffolds for Dermal Reconstruction To Predict In vivo Outcome

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The commercially available collagen-based dermal reconstruction scaffolds Integra® (I) and Matriderm® (MD) are not widely used in major trauma because of variable integration and equivocal outcomes. Smart Matrix (SM) is a new fibrin based scaffold which shows rapid integration and vascularisation in vivo. The aim of this study was to create a novel in vitro model of 3D cell ingress into dermal scaffolds to predict in vivo outcome. Cell proliferation, apoptosis, matrix contraction, cytokine/growth factor profile, α-smooth muscle actin (α-SMA) expression and cell morphology were studied for SM, I & MD.

Normal primary human dermal fibroblasts from 3 donors were cultured on coverslips (C-), contractile collagen gels (CCGs, C+ in apoptosis), I, MD and SM for 2 or 7 days. The 3 dermal scaffolds did not contract over time. Cell proliferation on SM was higher than on the collagen matrixes, corroborated by Ki67 immunostaining. Morphology (cross-sections) showed marked differences: cells in SM were elongated compared to stellate in CCGs or cuboidal in both I and MD, confirmed by α-SMA immunostaining. Electron microscopy showed cell-matrix adhesion on all the scaffolds. Apoptosis levels were very similar between the 3 dermal scaffolds. Cytokine/growth factors profile was similar for all the scaffolds, except in SM vascular endothelial growth factor (VEGF) increased, tumor necrosis factor α (TNFα) & transforming growth factor β (TGFβ) decreased.

In conclusion, these differences in proliferation, α-SMA and cytokine profile between scaffolds using the in vitro model are indicative of the extent of in vivo integration response rather than quantitatively representative. Particularly pro-angiogenic and cellular properties of SM are demonstrated.
Hydrogels with high water contents are often used in wound healing dressings. We have shown that high MW extracts of KGM (a heteropolysaccharide of glucomannan) of *Amorphophallus* significantly stimulate fibroblast proliferation. The aim of this study was to develop a biologically active hydrogel for wound healing. We synthesized a hydrophilic semi and full interpenetrating network (IPN) of KGM with poly(N-vinyl pyrrolidinone) crosslinked with poly(ethylene glycol diacrylate) by photopolymerization. Altering the percentage of KGM and initiators used in the polymerization affected chemical, physical and biological properties of the hydrogels. We measured equilibrium water content and quantified the presence of bound water in the hydrogels using differential scanning calorimetric (DSC) and found that semi IPN had the highest EWC (>90%) and bound water equivalent to native KGM compared to full IPN (>80%) and plain hydrogels (>70%). Semi IPN also had the highest content of KGM as measured by toluidine blue staining and solid state NMR. Proliferation assay showed that full IPN and soluble KGM are equivalent (180% and 200%) in their ability to stimulate fibroblasts while semi IPN did not. This work introduces a class of biologically active polysaccharide based hydrogels that may be useful for wound healing applications.
Piezoelectric PHBV Bilayer Construct as an Innovative Skin Substitute

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Being piezoelectric, the natural and biodegradable polymer polyhydroxybutyrate-co-hydroxyvalerate (PHBV) has the capability of generating electrical charges in response to mechanical strain. Therefore, this work intended to explore this property by creating an innovative skin tissue engineered substitute capable of improving wound healing. Bilayered PHBV scaffolds were prepared by combining solvent casting and freeze-drying methodologies, to produce a thin membrane with microporosity and a 3D porous structure recreating the epidermal and dermal layers, respectively. The combination of the two structures allowed attaining a scaffold with adequate mechanical properties to withstand the stresses that occur during the deposition of fibrotic tissue, thus diminishing wound contraction. The bilayered scaffold was totally degraded after 8 weeks in the presence of lipase and lysozyme at physiological concentrations. The human keratinocytes (hKc) and human dermal fibroblasts (hDFb) homotypic cultures, in the respective layer of the bilayer scaffold, confirmed the adhesion and proliferation of the cells along the time of culture. When co-cultured, both hKc and hDFb colonized the respective support structure depicting their characteristic phenotypes. Interestingly, after achieving confluence, hKc were able to grow in different layers as a stratified-like epidermis expressing involucrin, a marker of Kc terminal differentiation, in the upper layer (Fig. 1).

![Involucrin/Phalloidin/DAPI](image)

Figure 1: Z-scan images of the epidermis-like structure showing involucrin (green) expression on the upper layer. Nuclei were stained with DAPI (blue) and cells cytoskeleton with phalloidin (red).

A bilayered skin substitute comprising biologically interactive dermal and epidermal analogs was successfully produced. Those properties, associated with the piezoelectricity character of the PHBV, represent a promising strategy to improve skin wound healing.
Negative effects of denervation on wound healing are common in clinic cases. Vascular endothelial growth factor (VEGF) is effective in phases of healing. Tissue engineering scaffolds are good alternatives in the healing of wounds. In this study, initially the rats were denervated and wounds were created on the back side of the experimental animals. Electrospun membranes, cryogel scaffolds and VEGF loaded cryogels were implanted on the wounded sides. Control side was left without any implantation. After 7 and 21 days of implantation the wounded sides were taken, sections which were stained with Hematoxylin-eosin and modified Masson Tricrom techniques were evaluated to score healing process. The 7 days macroscopical results showed that the integration of the cryogel scaffolds was higher than the electrospun scaffolds. The changes of the wound surface area was also measured. At the end of 21 days, wounds in all groups were mostly closed. The results and comparison of the wound healing profiles of control and scaffold implanted groups will be presented in this presentation.
Mimicking Stem Cell Niche Environment To Enhance HFSC In vitro Expansion

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Human hair follicle outer root sheath (hORS) cells are known to contain hair follicle stem cells (HFSCs) and play an important role in healing large size wounds, and thus can serve as the cell source for skin engineering. This study investigates the effect of low oxygen and clonal expansion on hORS cell proliferation. Spare scalps were donated by the patients undergoing hair transplantation and freshly isolated scalp hORS cells expressed both CD200 and CK15. Further, hORS cells proliferated much faster in 4%O₂ than in 21%O₂ with 23 folds more cell yield, better cell colony forming efficiency (CFE) and higher levels of CD200 and CK15. hORS cells expanded in low oxygen generated much better quality engineered skin compared to cells expanded in 21% O₂. With low seeding density, hORS cells were able to form cell clones and these cell clones were harvested and re-seeded on the culture dish in low density. This clonal expansion method was able to generate 95 folds more cell yields than regular culture method and could engineer better skin than their counterpart. Interestingly, there was synergistic effect between low oxygen culture and clonal expansion methods. In conclusion, clonal expansion under low oxygen tension can mimic stem cell niche environment to better retain the stemness of contained HFSCs of cultured hORS cells, which not only increases the cell yields but also enhances the cell function for generating better engineered skin.
Investigating Decellularisation Protocols for the Production of Small Intestine Submucosa (SIS)

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A number of medical conditions including atresia and cancer can result in the requirement for extensive oesophageal surgery or transplantation. Tissue engineering represents an alternative to this. It was hypothesized that the use of small intestine submucosa (SIS) would represent a good starting scaffold material though it suffers from poor mechanical properties due to early remodelling. SIS is decellularised using peracetic acid under agitation and it was investigated whether the use of two alternative detergent-based perfusion decellularisation methods might represent improvements to the mechanical properties while also performing adequate levels of decellularisation. The samples were analysed by mechanical uniaxial tensile testing, DNA quantification, SEM and light microscopy. The results indicate that that in terms of desirable mechanical properties, failure strain and yield stress, the samples found to be SDS/Triton X-100 > peracetic acid > control > SD/DNase. DNA data showed the detergent based methods having levels below 50ng/mg dry weight (a proposed level indicating adequate decellularisation) with the SDS/Triton X-100 being the lowest. Peracetic acid produced SIS with DNA levels similar to the control. Light microscopy (H&E) confirmed the DNA results. It was concluded that due to effective decellularisation and the promising mechanical properties that the SDS/Triton X-100 actually produced the most desirable SIS. It was speculated that one possible reason for the poor decellularisation of the peracetic acid SIS was due to the tubular shape of the tissue did not allow for ideal decellularisation under agitation. This will be investigated in future work. The SDS/Triton X-100 protocol will be used to produce SIS which will be modified by the addition of a polymer layer for the purposes of in vivo oesophageal tissue engineering.
The Development Of A Novel Human Corneal Substitute Using Decellularized Corneas

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There is currently no reliable or standardized protocol for decellularization of human corneas. The discrete integration between corneal architecture and functional integration is vital to maintaining the native (keratocyte) cell phenotype in vivo and this inevitably affects cell type in vitro. Decellularization protocols need to sufficiently eliminate cellular material with minimal disruption to tissue architecture. Additionally a technique needs to be established for successful cell infiltration. The aim is to utilize corneal eye-bank tissue deemed unsuitable for transplantation via optimized human specific decellularization techniques and recellularization using novel, enriched, corneal stem cell populations which may be utilized in two key areas: (i) A more relevant human corneal substitute for drug and irritant testing in order to replace animal work and (ii) the creation of an effective engineered corneal construct for corneal transplantation. Removal of detectable cellular and immune reactive material will be evidenced by immunofluorescence and CFSE based mixed lymphocyte assays. Preservation of biomechanical, dynamic biomechanical analysis, optical properties and retention of corneal architecture will be assessed. The vision is that this research will yield reproducible, reliable constructs, available on demand. From an international public health standpoint, and from the perspective of patient quality of life, there is an undeniable need to develop a reliable artificial and healthy biomimetic cornea. We endeavor to provide the underpinning research to demonstrate that a bioengineered cornea repopulated with normal stromal and epithelial cells is both possible and clinically viable.
In-vivo Chimeric Human-Swine Arterial Implantation to Investigate the Fate of Seeded Cells on Decellularized Scaffolds

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Cardiovascular diseases are the leading cause of death in western countries; current clinical substitutes don’t perform well on long term patency for under 6mm diameter substitutions. Decellularization is the removal of all cellular and nuclear matter from a tissue leaving the extracellular matrix as intact as possible. This technique is being used in various tissue engineering (TE) fields (as blood vessel substitutions, BVS) since the not immunogenic and mechanical-matching substrate obtained with the decellularization. The current state of the art in BVS is shifting away from the classical TE paradigm (cells, scaffold and bioreactor) towards an unseeded approach that uses the body as both cells source and bioreactor; in this field, the aim of the current study is to investigate if seeded cells do not detach after an arterial in-vivo implantation. A detergent-enzymatic decellularization protocol was designed and validated on both biological (cell removal, residual DNA) and mechanical (compliance, burst pressure) properties in order to obtain the decellularized scaffolds from swine donors. Vessels were seeded with human endothelial and mesenchimal cells. We performed implants in swine models (Sus Scrofa 25kg weight) as iliac artery replacement (length 5cm), animals were immunocompromised through drug therapy (0.6ml/day cyclosporine) and sacrificed at 14 days. Results showed a patent vessel with endothelial layer (vWF+) and smooth muscle cells (aSMA+), interestingly there was no evidence in the lumen of residual human seeded cells (humanCD31+) but cells were from the host (swineCD31+). In conclusion these preliminary results suggest that seeded cells detach from the lumen after implant but may play a role in short-term patency.
In this work we investigated whether osteoclastic cells derived from human peripheral blood co-cultured with devitalized hypertrophic cartilage (HC) produced in vitro by adult human mesenchymal stromal cells can induce matrix remodeling into bone tissue. HC was devitalized by successive freeze/thaw cycles and cultured alone as control or together with freshly isolated CD14+ monocytes in the presence of osteoclastogenic factors (MCSF; RANK-ligand) for up to 23 days. Moreover, after 1 day of in vitro culture, samples from both groups were implanted ectopically in nude mice for 8 weeks. Analysis consisted of biochemistry, protein assays, histology and microtomography. In vitro results indicated matrix degradation through a significant loss of glycosaminoglycans only when HC was cultured with osteoclastic cells. In addition, supernatants of co-cultures contained significantly higher amounts of chemoattractant (MCP-1 192-fold; SDF-1 4-fold), angiogenic (IL-8 556-fold; VEGF-A 5.4-fold) and matrix degrading (MMP9 13534-fold; MMP13 8.5-fold) factors as compared to controls. Only co-cultured HC generated frank bone through endochondral ossification in vivo, with a 3.5-fold higher mineralized volume as compared to controls. Collectively, the addition of osteoclastic cells on devitalized HC primed the onset of remodeling leading to bone formation. Ongoing experiments aim to identify some crucial factors released from the HC matrix in co-cultures which are capable to recruit key cell populations involved in ossification.
Functional vascularization is a prerequisite for *in vitro* engineered tissues in order to be viable *in vivo*. However the *in vitro* engineering of 3D tissues with complex vessel networks and a functional endothelium still remains a major challenge. Hence, there is a need for standardized models recapitulating the endothelial microenvironment *in vitro* that will allow us to study and better understand vascular biology in order to overcome current limitations. Whole rat hearts were decellularized preserving macro- and microscopic architecture as well as biomolecular integrity, as previously described. The coronary vessel system of the decellularized hearts was cell seeded by retrograde aortic perfusion and the re-populated hearts were subsequently processed into two standardized tissue-flaps adherent to the aorta ascendens along the LCA and RCA, respectively. Coronary vessels of the tissue-flaps were successfully repopulated with different endothelial cell types, such as HUVEC and human ECFC cells, leading to an endothelialized vessel system with retained patency. Repopulated tissue-flaps were selectively cannulated through the coronary ostia for selective blood perfusion of the re-endothelialized vessel system. The here presented cardiac ECM vessel model provides a platform for studying ex vivo hemocompatibility and endothelial function of different donor endothelial cell types in a standardized, high-volume producible and reasonably downscaled vascularized cardiac ECM *in vitro* model with high biological fidelity. Such a native derived ECM vessel model might help us to overcome current limitations regarding the functional vascularization of engineered 3D tissues.
Three-Dimensional Kidney Scaffolds for Renal Tissue Engineering

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Chronic renal disease is a progressive condition marked by deteriorating kidney function leading to kidney failure, for which the only treatment is dialysis or organ transplantation. To reduce access to dialysis and address transplant organ shortage, a promising strategy is to regenerate damaged kidney using tissue engineering technologies. The aim of the present study was to produce a 3D rat kidney scaffold by a decellularization process and to repopulate the scaffold with stem cells using a dynamic perfusion method. Histochemical analysis showed that rat kidneys were successfully decellularized after 17 hr perfusion with sodium dodecyl sulfate. The whole-kidney scaffolds preserved the threedimensional architecture of vessels, glomeruli and tubuli as shown by TEM and SEM. Micro-CT scan confirmed the integrity and patency of the vascular network. Immunofluorescence demonstrated preservation of extracellular matrix proteins, including collagen IV, laminin and fibronectin. A series of n=11 experiments demonstrates that twenty-four hours after infusion of kidney scaffolds with murine embryonic stem cells through the renal artery and perfusion with recirculating cell medium, the seeded cells were almost completely retained into the organ and uniformly distributed in the vascular network, arteries, arterioles and glomeruli. Some cells reached peritubular capillary and were localized within tubular compartment. Our findings indicate that rat kidneys can be successfully decellularized to produce renal extracellular matrix scaffolds in a relatively short time and that a recellularization of vascular structures and glomeruli is achievable with dynamic perfusion at physiologic pressure. This study provides a model for the study of new strategies for regenerative medicine of renal tissue.
Decellularization of Large Tendon Structures: A Comparison of Protocols

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Finding a suitable scaffold for tendon tissue engineering is still a major challenge. Decellularization of native tendon tissue leads to a scaffold which is comprised of natural extracellular matrix and still displays the same mechanical properties as tendons. However, controversy exists over the optimal decellularization protocol. The aim of this study was to compare combinations of different physical (repetitive freeze-thaw cycles or incubation in hypotonic solution) and chemical (incubation in 1 % Triton-X or 1 % sodium dodecyl sulfate (SDS)) approaches for decellularization of tendons. Using large equine flexor tendons, the effectiveness of cell and DNA removal, the preservation of the extracellular matrix and the cytocompatibility of the obtained scaffolds were quantified and compared. While all protocols led to significant removal of cells and DNA compared to the controls (p>0.1), protocols including freeze-thaw cycles were more effective than those without freeze-thaw cycles (1 % vs. 20 % residual nuclei and 20 % vs. 40 % residual DNA) (p<0.5). Transmission electron microscopy further showed that decellularization did not lead to morphological extracellular matrix alterations. Scaffolds could be re-seeded with superparamagnetic iron oxide labeled, multipotent mesenchymal stromal cells. The labeled cells could be visualized by histology and magnetic resonance imaging and were oriented in alignment with the collagen fibers of the scaffold. There was a tendency that cell distribution into the scaffold was better when Triton-X had been used for decellularization instead of SDS.

Based on our results, we recommend to combine freeze-thaw cycles and treatment with Triton-X for decellularization of large tendon structures.
Pressurised Transmural Tracheal Decellularisation for Tissue Engineering Purposes

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Tracheal transplants are not commonly performed as the benefits rarely outweigh the risks. Tissue engineered airways offer a potential avenue for patients’ suffering from tracheal trauma and have achieved success in clinic, but there remain concerns about the short-term loss of biomechanical properties, necessitating the need for stents. In this study, pressurised transmural tracheal decellularisation was developed to investigate whether the biomechanical properties of the scaffold could be improved whilst achieving full decellularisation in substantially reduced time. Porcine tracheas were treated with detergent and nuclease washes in a bioreactor designed to generate pressurised transmural flow, at 0.35 bar, over a 4 day period. The results were evaluated by histology, ECM component quantification and by biomechanical strength testing. The results were compared to native trachea and trachea decellularised with the clinically used method (a 28 day process). Early results indicate improved cell clearance, most notably in the cartilage rings and a corresponding significant improvement in residual DNA removal. Glycosaminoglycan and collagen levels, important ECM components for biomechanical strength, were retained in the scaffolds produced using transmural pressure to a comparable level as the clinically used method. Scaffolds produced using transmural pressure retained biomechanical strength comparable to native trachea, whilst the current clinical method produced a significantly weaker scaffold ($p < 0.05$). Decellularisation using transmural pressure offers an alternative to the clinically employed decellularisation method due to comparable, if not improved, scaffold strength and reduced processing time.
Evaluation of the ECM Optical Properties of a Cornea Substitute Based on Decellularized Scaffolds

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Decellularized xenografts have been used as bioscaffolds in cornea tissue engineering. Ideally, the decellularization process should remove cells, while preserving the extracellular matrix (ECM) components. In this work, we evaluated a physical decellularization method based on sonication. Tissue specimens were subjected to sonication for 10 min, 20 min and 30 min submerged in buffer. Then, samples were washed three times in PBS for 48h. Cell removal efficiency and the ECM preservation pattern were histologically and histochemically analyzed and quantified in decellularized cornea substitutes. To evaluate the optical properties of these tissues, transmitted light was calculated by using a spectrophotometer. Our results revealed that 95.65% to 97.91% of the tissue cells had been efficiently removed from decellularized specimens using sonication methods in all times (p<0.05 as compared with the control tissues). However, our histological analysis showed an alteration of ECM fibrillar components, especially collagen and reticular fibers. Non-fibrillar proteoglycans and glycoproteins were mostly preserved. Decellularized tissues showed higher transmittance than control uncellularized specimens with an increase of 30 to 45%. These results suggest that sonication protocols are highly effective for cell removal and to preserve non-fibrillar components. Therefore, this decellularized tissue model could be applied to cases when the cornea stroma, mainly formed by collagen fibers, is not deeply damaged and an eye surface substitute is in need.

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Enabling the Induction of Neovascularisation in Tissue-Engineered Skin

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Introduction: Delays in developing new vasculature after tissue transplantation is a major problem in getting tissue-engineered (TE) skin to survive on the patient's wound bed. To overcome this we aim to induce neovascularisation in TE skin using a bioreactor and 'pre-vascularised' skin, obtained by incorporating TE skin with a re-endothelialised biological vascular network. Here we discuss the decellularisation of rat liver and jejunum to establish the networks.

Rat liver and jejunum were harvested from fresh cadavers. The inferior vena cava of the liver and main vessel of the jejunum were cannulated before flushing with heparin solution. Distilled water was perfused through the organs at 5 mL/min for 3 hours. Subsequently, 1% Triton-X 100 with 0.1% Ammonium Hydroxide was perfused for 12 hours. Distilled water was then circulated to remove residual detergent. Blue dye was injected into the organs to assess vascular patency. Low magnification and confocal microscopy along with microCT imaging were used to further examine the networks. Histochemical and DAPI staining were used to characterise organ composition.

A well-defined vascular tree with multiple branching was visible from blue dye injection and imaging techniques. Histochemical analysis showed staining expected from proteinous ECM but no evidence of cells (Fig. 1). The latter point was confirmed with DAPI staining.
Towards Engineering Vascularized Adipose Tissue Utilizing Decellularized Porcine Jejunal Segments and a Custom-made Bioreactor System

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In adipose tissue engineering for reconstructive and plastic surgery, adequate vascularization of large constructs still remains a crucial challenge. Decellularized jejunal segments represent a potential scaffolding system in which pre-existing capillary structures can be reconstructed, thus, possibly enabling the development of large vascularized tissue constructs. Therefore, objectives of this study were a) to reseed the capillary structures of decellularized porcine jejunal segments with microvascular endothelial cells (MVEC) utilizing a custom-made flow-through bioreactor system, and b) to seed the jejunal lumen with adipose-derived stem cells (ASC) and induce adipogenic differentiation of the stem cells in this co-culture system.

The capillary structures of the decellularized jejunum (length 8 cm) were seeded with 6*10⁶ MVEC and cultured in a medium based on VascuLife (CellSystem). Successful repopulation of the vasculature was shown by histology and MTT assay for living cells (day 14). After two weeks of culture, the lumen of the jejunum was seeded with 5*10⁶ ASC. The whole construct was then cultured in a previously established coculture medium based on PGM (Lonza) and VascuLife. Immunohistochemical staining for CD31 indicating MVEC and haematoxylin staining for cell nuclei demonstrated a vascularized stem cell-laden construct (day 21). Adipogenic induction was then induced on day 21 and continued for three weeks. Adipogenesis and vascular ingrowth were observed up to day 42 through histology, immunohistochemistry, whole mount staining, and qRT-PCR. In on-going work, the culture conditions are varied to optimize the vascularization and the adipogenic differentiation within the adipose construct.
Feasibility of Radiosterilized Amnios as an Alternative Scaffold for the Treatment of Burn Patients

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Burns have become more frequent nowadays. Patients with this condition are currently treated with a biological wound dressing sterilized with gamma radiation, called amnios. Amnios is the innermost of the three layers forming the fetal membranes and in this study it is used as a scaffold, having considerable interest for tissue engineering applications. The aim of this study was to evaluate attachment, viability and growth of cells seeded in radiosterilized amnios, in order to create a new construct and provide wound coverage to burn patients. A total of 5 human skin biopsies were obtained from plastic surgery. They were enzymatically digested. Dermis was separated, followed by isolation of dermal fibroblasts. Cells were cultured in standard conditions using appropriate medium. Cells were seeded onto the amnios, placed in the bottom of the cluster. After three weeks we tested cell viability, immunophenotype and attachment by SEM. Fibroblasts attached and proliferated, producing a typical extracellular matrix (ECM) when they were cultured onto the radiosterilized amnios. We observed that cells were stimulated by this natural scaffold, indicating that cells interact with it. Immunohistochemical analysis showed positive cytokeratin 19 and vimentin cells. Amnios affords cell adhesion, promotes cell proliferation and production of ECM, contributing to make a novel skin substitute with natural properties. This construct is an alternative suitable natural scaffold for the treatment of burn patients and might be useful to burn patients in the Mexican population.
Skeletal Muscle Tissue Formation Potential of Acellular Skeletal Muscle Originated Native and Semi-Synthetic Scaffolds Loaded with Human Myoblasts and Mesenchymal Stem Cells

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Currently, skeletal muscle tissue defects are treated by transplanting a graft from another part of the patient’s own body, but this results in donor site morbidity in case of large defects. With the aim of providing an alternative treatment approach, skeletal muscle tissue formation on synthetic (Poly(L-lactide-co-caprolactone) (70:30)), semi-synthetic and acellular native scaffolds loaded with human myoblasts and menstrual blood derived mesenchymal stem cells (hMB-MSCs) was investigated in this study. Human myoblasts were isolated by enzymatic digestion from a small skeletal muscle biopsy, and hMB-MSCs were isolated by their plastic adherence property. The cells were characterized through immunostaining and differentiation into osteogenic and adipogenic lineages. The synthetic parallel fiber scaffolds (fiber dia: 4.53 ± 1.6 micrometers) were produced by electrospinning. Native scaffolds were obtained by detergent treatment of the human muscle tissue. Semi-synthetic scaffolds were produced by cross-linking acellular muscle particles to the electrospun synthetic scaffolds. The myoblasts and hMB-MSCs at passage 4 were cultured on the scaffolds for 21 days and cell proliferation assay (WST-1) and SEM imaging was carried out at the end. hMB-MSCs did not grow on the untreated synthetic scaffolds. Scaffolds seeded with myoblasts and incubated for 14 days were stained for alpha-actinin to study myotube formation. We conclude that native acellular and synthetic scaffolds and myoblasts as a cell type were effective in producing a muscle tissue patch. Producing oriented muscle tissue substitute was more successful when synthetic scaffolds and myoblasts were used.
Decellularized Porcine Trachea for the Reconstruction of a Bronchial Respiratory Model In vitro

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In vitro coculture models mimicking the bronchial barrier are a significant step forward in investigating the behaviour and function of bronchial epithelial cells. Up to now only synthetic materials were used as substrates. Decellularized tissues provide a more in vivo-like environment based on the native extracellular matrix. Therefore, an in vitro bronchial coculture model has been established using a decellularized porcine luminal trachea membrane and employing 3 relevant human cell types. The tissue was decellularized and placed in plastic transwell supports. The bronchial epithelial cell line 16HBE14o- was seeded on the apical side of the membrane and the lung fibroblast cell line Wi-38 and/or the microvascular endothelial cell line ISO-HAS-1 were seeded on the basolateral side. TER (Transepithelial Electrical Resistance) was measured over a week and tight/adherens junctions (ZO-1, occludin/β-catenin) were assessed via immunofluorescence. In the cocultures the response of ISO-HAS-1 to LPS (lipopolysacharide) was examined by E-selectin ELISA. Cultures successfully grew on the membrane. TER values of around 521±120,76 Ω*cm² were achieved in the monoculture and 641±143 Ω*cm² in the cocultures. ZO-1, occludin and β-catenin were expressed in 16HBE14o- in all conditions but were even higher in the cocultures. Endothelial cells responded to the LPS stimulation by producing soluble E-selectin. Conclusion: Fibroblasts and endothelial cells support the formation of a tight barrier by the 16HBE14o-, probably as a result of paracrine communication with the cocultured cells. In summary, decellularized tissues are suitable materials to create an in vitro multicellular bronchial barrier.
Identification and Standardization of Neurotrophic Factors in Porcine Small Intestinal Submucosa

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Small intestinal submucosa (SIS) is a natural degradable biomaterial derived from the small intestine of vertebrates. Porcine small intestinal submucosa (SIS) has been widely used in repairing various tissues and organs, especially in nervous system. However, little is known about the bioactive materials within this material. In this study, we proposed the isolation method to get an acellular SIS then major growth factors which are able to regulate neuronal development within SIS accompanying with decellularization was quantitatively assessed by ELISA. Contamination of cell in SIS was evaluated by H&E and the content of genomic DNA. Acellularised SIS was obtained by sequential procedures; mechanical disassociation, degrease, enzyme digestion, freeze-drying, freezer-mill, extraction, concentration and filtration. BDNF (brain-derived neurotrophic factor), erythropoietin, GDNF (glial cell line-derived neurotrophic factor), NGF (nerve growth factor), and PDGF (platelet derived growth factor) discovered the components of SIS.
Use of biological materials in cardiac surgery is very common. However, the chemically treated prosthesis materials short-lived due to calcification and were required additional therapy. Therefore, the aim of our study was to investigate the biological and mechanical properties of decellularized arterial vessels and to evaluate the possibility of graft repopulation with MSC. MSC were obtained from bone marrow specimens using Mesencult-XF reagents thru 4 passages. Porcine arteries harvested and were treated with 1% sodium dodecysulfate (group A); 1% sodium deoxycholate (group B); and combination 0.5% SDS + 0.5% SDC, group C (n=5 each). After each of 10 wash cycles, samples of washing solutions were collected. Metabolic activity of MSC was assessed in the presence of washing solutions using cytotoxicity and metabolic assays (lactate dehydrogenase level and MTS test). Decellularized and washed arteries were reseeded with MSC. Histologic analysis showed sufficient decellularization of tissues in all groups. But the best preservation of the connective tissue structure and the absence of any cells were achieved only in group C Only after the 4 and 6 washing cycles detergents level had no cytotoxic effect on the metabolism of human MSC at groups C and A,B respectively. 10-colour flow cytometry examination showed more than 97% purity level of cultured MSC. Decellularized and washed scaffolds were reseeded with human MSC at density 2-5*10^5 cells/sm. After one week of cultivation in static conditions, evenly distributed cells were observed on the graft surface. It was tried to form cobblestones-like structures. Individual cells penetrate beyond the basement membrane.
Preparation of Acellular Porcine Renal Scaffold for Kidney Regeneration

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The native extracellular matrix (ECM) with intact 3D anatomical architecture can be a promising alternative to synthetic scaffolds. In this study, we have developed an acellular porcine renal scaffold, analyzed its physical characteristics and biocompatibility for kidney regeneration. A wedge shape porcine kidney cortex was obtained and treated with 1% (v/v) Triton X-100 or sodium dodecyl sulfate (SDS) for 72 hrs in a shaking chamber, and rinsed with PBS. After confirmation of decellularization using H&E stain, the matrix was sterilized and liophylized. The water absorption ability of the Triton X-100 treated scaffold (S-triton) was slightly higher than that of SDS treated one (S-SDS). The maximum compressive strength of S-triton and S-SDS were 0.1663±0.0297 and 0.3128±0.0382 Mpa. In vitro degradation rate of S-triton was significantly quicker than that of S-SDS. In FTIR analysis, both S-triton and S-SDS showed strong peak at 3445-3446 cm⁻¹ which belongs to amide II (–NH). For a biocompatibility analysis, primary human kidney cells were seeded on a scaffold. At 24 hrs post-seeding, adhered cells were 4.96 times denser in S-triton than S-SDS. On a cck-8 analysis, the S-triton showed significantly higher cell viability and proliferation rate than that of S-SDS. Histologic analysis 8 weeks after implantation of S-triton in a partial nephrectomized mouse, glomeruli formation was found in a graft close to host tissue. In conclusion, we could prepare an acellular porcine renal scaffold having proper physical, biochemical, biocompatibility characteristics for kidney regeneration.

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Stress urinary incontinence (SUI) is related, among others, with the malfunction of the closure mechanism of the rhabdosphincter, which is constituted by skeletal muscle cells. There are several in vivo models for SUI, but no in vitro model has been developed so far. The aim of this study is to establish a reliable in vitro model of rhabdosphincter for the development and screening of new drugs and therapies for SUI. Piglets’ urethras were decellularized using different concentrations of Triton X-100 and sodium dodecyl sulfate (SDS) and a system based on agitation (60 rpm) and perfusion (40 mL/min). The efficiency of cell removal on decellularized urethras was assessed by Hematoxylin & Eosin (H&E) staining and confirmed by DNA extraction. The presence of collagens I-IV, elastin, fibronectin and laminin was evaluated by immunofluorescence staining. Total collagen, elastin and glycosaminoglycans were also quantified by specific colorimetric assays.

0.5% and 1% SDS solutions successfully decellularized the urethra, as shown by H&E, which revealed no purple basophilic staining (nuclear material). 0.5% SDS removed 93.4±2.6% of DNA material, while 1% SDS removed 96.1±0.0%. Immunofluorescence staining indicated no major loss or modification of extracellular matrix (ECM) proteins localization; however, the presence of elastin and fibronectin in the acellular ECM was lower compared to native tissue. Quantification of the total amount of ECM proteins showed no major differences between native tissue and acellular scaffold.

Our results indicate that dynamic decellularization of the urethra was able to remove up to 93% of the total DNA while preserving ECM chemistry and localization at the lowest concentration of SDS tested. Future studies include recellularization, where cell adhesion, proliferation and most importantly, recovery of rhabdosphincter function, will be assessed.
Rapid Transplantation with a Levitation Device for an Epidermal Cell Sheet in a Porcine Endoscopic Submucosal Dissection Model

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To date, regenerative medicine using cell sheets has been successfully established for replacing a dysfunction tissue. After the endoscopic resection of mucosal epithelia in an esophagus, the transplantation onto the removed part with autologous oral mucosal epithelial cell sheets has promoted the early wound-healing of mucosal epithelial defect region. Furthermore, a cultivated epidermal cell sheet also has a wound-healing potential. However, in these methods, the highly severe manipulation of endoscope has been required to transplant a cell sheet through a narrow digestive tract, so that there has been the issue of the extension of surgery time. Therefore, this study aimed at the improvement of surgery time by using a device for cell-sheet transplantation. The device which has three air nozzles was developed by using a rapid prototyping system (Stratasys, Rehovot, Israel). A cultivated porcine epidermal cell sheet was prepared. After the endoscopic submucosal dissection, the epidermal cell sheet was transplanted by using a supporting membrane made of polyvinylidene difluoride (PVDF) or the developed device. A transfer time from oral section to transplantation region and a transplantation time were statistically analyzed. The analyzed time was as follows (average +/- standard deviation). PVDF method (n = 3): 238 +/- 104 s, for the transfer time; 199 +/- 72 s, for the transplantation time. The device method (n = 7): 87 +/- 55 s, for the transfer time, 66 +/- 30 s for the transplantation time. Both transfer and transplantation times in case of using the device were less than the half of that in case of using the PVDF supporting membrane. Therefore, the device would be an essential instrument for reducing the surgery time of endoscopic transplantation of cell sheet in regenerative medicine.
We exploited the biomimetic approach to generate constructs composed of synthetic biphasic calcium phosphate ceramic and extracellular matrix (SBC-ECM) derived from adult human dermal fibroblasts in complete xeno-free culture conditions. The construct morphology and composition were assessed by scanning electron microscopy, histology, immunohistochemistry, Western-Blot, glycosaminoglycan and hydroxyproline assays. Residual DNA quantification, endotoxin testing and local inflammatory response after implantation in a rat critical-sized calvarial defect were used to access the construct biocompatibility. Moreover, in vitro interaction of human mesenchymal stem cells (hMSCs) with the constructs was studied. The bone marrow- and adipose tissue-derived MSCs were characterized by flow cytometry and tested for osteogenic differentiation capacity prior seeding onto SBC-ECM, followed by ALP, MTT assay and real-time qPCR to assess the osteogenic differentiation of hMSCs after seeding onto the constructs at different time intervals. The SBC-ECM constructs enhanced osteogenic differentiation of hMSCs in vitro and exhibited excellent handling properties and high biocompatibility in vivo. Altogether, our results highlight the ability to generate in vitro fibroblast-derived ECM constructs in complete xeno-free conditions as a step towards clinical translation, and the potential use of SBC-ECM in craniofacial bone tissue-engineering applications.
Evaluation of Ionic and Non-Ionic Detergents as Cornea Decellularization Agents

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Xenogeneic cornea decellularization could be a proper source of corneal stroma scaffolds. However, there is no consensus about the most adequate decellularization protocol for the animal cornea. In this work, we have evaluated several decellularization protocols based on ionic and non-ionic detergents on porcine corneas. Porcine corneas were obtained from a local slaughterhouse. After washing the corneas with PBS and antibiotics, 4 types of detergents were evaluated as decellularizing agents: sodium dodecyl sulfate (SDS), Igepal, Triton X100 and benzalkonium chloride (BAK) using different concentrations (0.01%, 0.05% and 0.1%) and times (12, 24 and 48 hours). Decellularized corneas were histologically analyzed to determine decellularization efficiency and collagen fibers preservation. First, histological analyses showed that endothelial cornea cells were efficiently eliminated by all agents; epithelial cells were removed with Igepal (48 hours – all concentrations), SDS (0.05% and 0.1% - all times) and triton X100 (24h and 48h – all concentrations); and stromal keratocytes were completely removed only by 0.05% SDS (24h and 48h) and 0.1% SDS (all times). The integrity of the collagen bundles was partially preserved with all the detergents used. These results show that the use of SDS represents the most accurate decellularization method for the porcine cornea when different detergents are compared. This method could have potential usefulness for the generation of a corneal scaffold for clinical and experimental uses.

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Decellularized Human Placenta Vessel Matrix for Tissue Engineering

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The decellularized matrix of the placenta has great potential for use as a scaffold for tissue engineering. One big advantage of this system is that the organ can be readily harvested without harm to the donor. The placenta is on one hand a rich source of human cells and also of human ECM components as well as vascular structures.

We developed a protocol to decellularize an intact segment of the human placenta chorionic plate in order to isolate a functional vessel loop. To facilitate the complete decellularization of the dense matrix, a system has been designed to perfuse the required chemicals into the placenta via the existing vasculature of the chorionic plate. Histological, immunohistochemical, and scanning electron microscopic analyses confirmed the removal of the cells and cellular debris and characterized the composition and structure of the matrix.

We are currently developing further protocols to optimize the decellularization steps. This should assure not to destroy too much of the extracellular matrix (ECM). Analyses to determine important structural components after the decellularization are ongoing.

The decellularized vascular network shall be preserved for a recellularization with cells. The combination of decellularized ECM and the vascularization process should lead to new approaches and techniques for organotypic models. This project is supported by the seventh frame network programme Biodesign (NMP.2010.2.3-1).
The manufacture of allogeneic human derived 3D scaffolds seeded with autologous cells is one of the approaches used in the tissue engineering field. For an urgent compassionate clinical case of a 15 year old female with congenital stenosis of the trachea, a pre-clinically validated 10 day vacuum-assisted decellularisation method was translated to cGMP compliance and used to decellularise a donor trachea. The patient’s Mesenchymal Stromal Cells (MSCs) and bronchial Epithelial Cells (EC) were expanded in vitro previously and cryopreserved until seeded onto the scaffold. MSC were obtained from isolation of mononuclear cell layer from patient’s own bone marrow, expanded and cryopreserved until needed. A 3-day differentiation protocol for MSC was used to obtain a chondrogenic cell lineage. EC were obtained from tracheal biopsies and expanded on dedicated medium. A custom-made bioreactor was used to seed the MSCs, differentiated MSCs and epithelial cells onto scaffold over a 4 day period. Prior to release of the product, histological results from the decellularisation confirmed the scaffold to be acellular and immunohistochemistry with anti-HLA-1 confirmed no immunogenic donor material to be present. Sterility was confirmed by aerobic and anaerobic BacTec culture throughout the procedure and microscopic analysis of the bioreactor medium immediately before release (on transplant day). The resulting ATMP TEP maintained an open and functional airway with no associated immune response further supporting the claim that decellularised/recellularised scaffolds manufactured as ATMPs are emerging as an alternative for airway transplants.
Biomaterials and the cell and tissue components are widely used in tissue engineering procedures for several decades. Currently, there is increasing tribute for understanding, screening and incorporating nanobiomaterials into medical applications. The nanoscale materials are candidates to be the complementary structures of the different cell types. This issue is of particular importance in the emerging field of regenerative medicine, the objective of which is to build up methods to repair, replace, and regenerate diseased, injured, or non-functional tissues. For this purpose, mesenchymal stem cells have been considered as a promising cell type, because of their expandability, and potential to be induced toward specific cell differentiation lineages.

The biology of mesenchymal stem cells, particularly the mechanisms regulating their proliferation versus differentiation into specific lineages, is regulated by cell-cell interactions, signaling by extracellular mediator molecules, and transcriptional or genetic mechanisms. The mesenchymal stem cells reside in bone marrow or stay as dormant cells within the adult connective tissues in order to provide tissue maintenance and mobilize and recruited to the injured inflamed or tumor environments. Their extracellular matrix milieu provides critical cues, both molecular and structure-dependent, to guide stem cell-mediated tissue responses. The biomimetic and biodegradable nano or micro scale biomaterials serving as scaffolds, labels or targets for cell-based tissue engineering can contribute to the stem cell action and may mediate its microenvironment.

This talk will address to the promises and the challenges in the field of regenerative medicine, related to imaging of the interaction of the mesenchymal stem cells and nanomaterial based scaffolds in their inflammatory microenvironment.
New developments with respect to imaging modalities in the clinical and pre-clinical arena introduce new challenges and possibilities with respect to image analysis and visualization. Specially in the pre-clinical arena the same specimen is scanned more and more with multiple imaging modalities to get most out of the same samples which are available. This multimodality scanning provides new challenges for multimodal imaging registration, image analysis, data extraction and visualization. Several steps in the whole pipeline of processing pre-clinical images and data will be presented including new upcoming modalities like the use of Optical Coherence Tomography (OCT) and matrix assisted laser desorption ionization imaging mass spectroscopy (MALDI-IMS).
Development of Large Animal Models from Veterinary Clinical Disease to Human Medical Preclinical Disease – the Contribution of Veterinary Medicine to Regenerative Medicine Exemplified Through the Development of an Ovine Tendon Defect Model

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Veterinary clinical disease can in several cases be used as a naturally occurring model for human clinical disease. Preclinical testing in large animal species being mandatory for clinical phase I testing of novel therapeutic strategies, such models can be very convincing. As an example, tendon disease in the horse is an excellent model for tendon disease in man. The use of horses for experimental purposes might, however, be too difficult to organize and therefore, the creation of an ovine model as another large animal model was targeted. The aim of this study was to develop an appropriate tendon defect model that permits to investigate the outcome of modern therapy concepts under standard conditions. The review of literature showed that numerous tendon defect models with very different issues have been applied into large animals, small animals as well as laboratory animals. Within the present study, known tendon defect models were examined with regard to the methodology used and the respective defect characteristics. The presented sheep model demonstrates a Core Lesion model. It simulates the most frequent tendinopathy occurring in horses and it offers the possibility to perform a direct intralesional injection of therapeutics. Such a model will in turn be useful to simulate similar lesions in human patients, demonstrating the role of clinical veterinary medicine for human clinical research and development.
The Andalusian Initiative for Advanced Therapies -a publicly funded organization created in 2008 by the Andalusian Government, Spain- was established to promote the development of new therapies with the view to improving the health of the population, driving innovation in our health care system and encouraging progress for our Region. To do this, we seek alliances with the academic world, research institutions, health centres, patients’ associations, SMEs and the pharmaceutical industry. Over the last few years, in collaboration with different Universities and Pharmaceutical companies, we have pushed forward the building of a cluster of research centres and institutes, biobanks and Good Manufacturing Practice (GMP) facilities. The Andalusian Initiative offers a distinct, major translational focus. Thus, our organization provides a comprehensive support hub to develop clinical research. Support comes not only in the form of financing, but also with implementation of complementary expertise (we have also designed, in collaboration with the University of Granada, a European Master Degree in Manufacturing of Advanced Therapies Medicinal Products). To summarize, we have built a network of 10 GMP facilities in multiple research centres, tissue banks and hospitals thus far. We currently support their accreditation and the development of investigational medicinal products, and the design and implementation of clinical trials. In the case of the latter, our support begins with a regulatory assessment during preclinical development, followed by the promotion of collaborations between basic and clinical researchers, and extended up to monitoring the safety of the resulting investigational medicinal products. As a result of this organizational system, we have already acted as sponsors of 18 Phase I/II clinical trials in the areas of cardiology, neurology, immunology, peripheral vascular disease and hepatic regeneration among others. At present we are collaborating with some biotechnological companies and other academic institutions in order to further development of these and other investigational medicinal products.
Novel Cyclized Polymers as a New Generation of High Effective Transfection Vectors

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Since the polyamidoamine dendrimer (PAMAM) demonstrated that its 3D hyperbranched structure could facilitate effective transfection, much effort has been turned to the preparation of branched versions of common transfection agents. Herein, we report the design and synthesis of new 3D ‘Single Cyclized’ polymeric gene vectors with well-defined compositions and functionalities via a one-step synthesis from readily available multi-vinyl monomers. The cyclization agent ethylene glycol dimethacrylate was used at 10% of monomer mixture, usually impossible due to insoluble gel formation were it not for the use of the highly controllable Deactivation Enhanced – ATRP synthesis method. This allowed single growing chains with PEG and a cationic monomer to crosslink within themselves forming a new structure for a transfection agent. This structure of cyclizing chains offers a different pattern of interaction between the polymer and plasmid DNA, and leads to a general profile of higher transfection capability (over 6 cell types) than SuperFect® (partially degraded PAMAM). By adjusting the monomer ratio of PEG, lower toxicity is also achieved, thus rendering the cyclised knot a more attractive alternative to the PAMAM dendrimer. Considering the simplicity of the synthesis, and ability to scale-up, these cyclized polymers show a profound advancement in the field of non-viral transfection.

Keywords: Non-viral, Transfection, Plasmid DNA, Polymeric gene vector, Cyclized polymer

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Heterogeneous Scaffold Design and 3D Bio-Printing for Macro-vascular Tissues

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Improvements in Additive Manufacturing (AM) processes for tissue engineering have enabled the development of porous scaffolds with interconnected and continuous pores. However, homogeneous scaffolds with uniform porosity do not capture the intricate spatial internal micro architecture of the replaced tissue. A novel heterogeneous scaffold modeling is proposed for layered-based additive manufacturing processes which generates scaffolds with functionally gradient porosity. Although there are several scaffold based studies about tissue engineering, there are some problems with cell to cell interaction, the assembly and alignment of ECM components and the host response to scaffolds. Therefore, especially vascular tissue engineering studies tend towards scaffold-free techniques. Novel computer aided algorithms and methods are developed for 3D printing of scaffold-free macro vascular structures. An example aorta model is generated using imaging and segmentation software. The developed algorithms are implemented using Rhinoscript. In order to support printed cell aggregates, support structures with ‘Cake’ and ‘Zigzag’ patterns are developed and 3D printed.

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Sol-Gel Derived SiO2 Matrices with Enhanced Mechanical Properties for Hard Tissue Regeneration

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Sol-gel derived SiO2 matrices are favourable materials for encapsulating different types of biologically active agents such as proteins, drugs and cells due to their biocompatibility and low temperature processing. These materials also have potential applications for bone regeneration due to the dissolution of silica and ion exchange with physiological fluid which induce formation of a calcium phosphate layer and the integration of implant to surrounding tissue. Application of these materials in tissue engineering, however, is limited because of low mechanical properties, leading to poor support for cells to adhere and proliferate. In this study, a class II hybrid was fabricated by chemical coupling of poly(methyl methacrylate) (PMMA) and silica matrix by using silane coupling agent (3-trimethoxysilyl propyl methacrylate (MPMA). Integration of PMMA chains into the structure of silica decreased gelation time from 5 days for silica to only 2 hours for hybrid by optimizing the composition of silica, PMMA and MPMA. Furthermore, the Young’s modulus decreased 40 fold and the hardness value increased from ~0.05 Vickers hardness (HV) for silica matrix to 33 HV for hybrid, which was in the range suitable for bone tissue engineering. Furthermore, it was observed that the presence of covalent bonding to PMMA chains, delayed the dissolution rate of silica 1.5 fold that resulted in prolonged ion exchange with physiological fluid. In vivo studies in mice showed that hybrids were superior in terms of maintaining their integrity and inducing a lower level of inflammation compared to silica. Therefore, the hybrid material fabricated in this study could be a superior alternative to the silica matrix for tissue engineering applications.
The Gap between Lab and Clinic

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Research results obtained in labs have to be verified in animal models and clinical studies before they are licensed as new products or medical devices for treatment in humans, if at all. There is a significant difference between interests and objectives of scientists in labs and those of surgeons in clinical application. This is also forced by the influence of the pharmaceutical and medical engineering industry. Regarding the realization process of a new product, these obstacles lead to important gaps between research results in the labs and application requirements in human treatment.

The above situation will be presented in different models of bone tissue engineering (bone-TE in critical sized defects and vertical bone development).

These techniques will be explained using groups of patients that show:
1. Critical sized defects, e.g. large cystic holes,
2. Total bone loss of the alveolar ridges,
3. Periodontal bone defects.

Bone tissue engineering techniques require:
1. Matrices,
2. Cells,
3. Signaling molecules

Keywords: bone tissue engineering, critical sized defects, bone loss
Biochemical Fingerprinting Cartilage – Non-invasive Raman Spectroscopic Analysis of Proteoglycans and Chondrocyte Differentiation State

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Proteoglycans (PGs) are important extracellular matrix (ECM) elements that are present in all tissues and organs. Pathological remodeling of these macromolecules leads to severe diseases such as osteoarthritis or rheumatoid arthritis. To date, PG-associated ECM alterations are routinely diagnosed by invasive analytical methods. Here, we employed Raman spectroscopy, a laser-based, marker-free and non-destructive technique that allows the generation of spectra with peaks originating from molecular vibrations within a sample, to identify specific Raman bands that can be assigned to PGs within human and porcine cartilage samples and chondrocytes. Based on the non-invasively acquired Raman spectra, we further revealed that a prolonged in vitro culture leads to phenotypic alterations of chondrocytes, resulting in a decreased PG synthesis rate and loss of lipid contents. Our results are the first to demonstrate the applicability of Raman spectroscopy as an analytical and potential diagnostic tool for non-invasive cell and tissue state monitoring of cartilage in biomedical research.
Signals in the Regenerative Niche to Accelerate Bone Growth

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Control of bone repair depends on understanding the nature of the regenerative niche. One of the options for studying this is the use of coculture systems with relevant cells, initially on tissue culture plastic and then on more complex 3D biomaterial scaffolds. As major limiting factors in bone regeneration are the speed and extent of vascularization, we have set up human osteoblast (pOB)-endothelial cell (EC) cocultures to study cellular crosstalk and its possible use for translational strategies. This presentation will give an overview of the present status, including novel, unpublished work.

The following approaches have been adopted:
1. Changing biomaterial properties, for example, polymer content in a polymer-ceramic composite.
2. Use of early embryonic signals, such as sonic hedgehog (shh) to accelerate both osteo- and angiogenesis.
3. Use of intermittent hypoxia, but not constant hypoxia, to promote vascular sprout formation.
4. Study of macrophage roles in the bone regenerative niche.
5. Possible application of pro-inflammatory signals, for example via stimulation of toll-like receptors (TLRs) to enhance the vascular response.

How this is investigated in coculture models will be presented and discussed in the context of future developments. Naturally, all phenomena from in vitro studies require proof of concept in relevant in vivo models, as only this approach can lead to a translational perspective.
This talk will focus on the design of nerve guidance conduits (NGC's) for the repair of peripheral nerve gap injuries, which are potentially advantageous as an alternative to the nerve autograft. However, current commercially available NGC's have a limited regenerative capacity to 5-10mm, partly due to the absence of physical guidance cues and a relatively poor ability to support nerve growth. Parallel work in the development of cell therapy methods based on Schwann cells and adipose-derived stem cells will also be considered, as such approaches can be combined with NGCs for enhanced therapeutic effect. The aims of the work are therefore primarily to: 1) design and fabricate micro-structured materials with improved physical properties; 2) develop better methods for primary Schwann cell therapy and 3) consider the suitability of adipose-derive stem cells for differentiation to a Schwann cell phenotype as an alternative approach. Degradable, photocurable polymers such as caprolactone are being used for 3D structuring by laser micro-stereolithography, which enables NGC devices to be fabricated rapidly with features not possible by traditional moulding techniques. In-vitro testing includes the culturing, cell viability testing and immunofluorescence labelling of neuronal cells, primary Schwann cells and dorsal root ganglia for differentiation marker analysis. Devices which show promise proceed to an in vivo mouse nerve injury model. The development of a novel method for primary Schwann cell culture will be presented, together with data on the potential of adipose stem cell differentiation to Schwann cells. In summary, the development of methods for peripheral nerve repair spans novel fabrication techniques for manufacturing NGCs, in combination with novel glial and stem cell methods for enhancement.
Bio-engineered, Clinically Applicable Skin Grafts: What Does it Mean to Produce them Under GMP Conditions?

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The two skin substitutes denovoDerm and denovoSkin, developed at the Tissue Biology Research Unit in Zurich, Switzerland, are to be tested in phase I clinical trials in the context of the FP7 funded EuroSkinGraft consortium in Zurich. In European Union and in Switzerland, the regulations for Advanced Therapy Medicinal Products (ATMPs) have changed markedly with the introduction of the regulation EC 1394/2007. After a transitional phase until end 2011, it now is obligatory for all ATMPs to comply with this regulation. For clinical trials, it has become mandatory to produce ATMPs under strict GMP conditions. Producing autologous skin grafts in a GMP facility is extremely demanding and poses a significant challenge for research teams that work in an academic setting. Prominent obstacles to overcome are the costs, which include the rent for a GMP facility, additional personnel and detailed in-process controls. For a production process that was previously perfectly established under experimental conditions, changes have to be made concerning GMP grade (or medical grade) starting materials, consumables and protocols. This means in most cases a time- and money consuming process of adaptation and testing. However, after all we are confident that we will succeed in coping with the “GMP-challenge” in collaboration with the regulatory authorities and for the sake and safety of our patients.
Cryogel biomaterials have emerged as an important field in tissue engineering and regenerative medicine. Tissue engineering applies the multidisciplinary concepts for the development of tissue substitutes which can regenerate the diseased tissue or organ. Thus it plays an important role in the area of regenerative medicine by restoring, maintaining or enhancing the tissue or organ function. Our work focuses on synthesizing the biomaterial materials from different polymers or polymeric precursors by a technology called cryogelation. These cryogel biomaterials have shown potential for application in scientifically challenging processes like, tissue engineering, cell separations, bioreactors for therapeutic protein production and extracorporeal devices. In the preparative and generic cell separation area we have designed an affinity based monolithic chromatographic approach for the separation of different cell types including stem cells. Other promising application of these supermacroporous matrices have been the cultivation of the mammalian cells on the support matrix for protein therapeutic production and for the design of a bioartificial lever support system. We have also explored the role of cryogel biomaterials as scaffolds for cartilage, skin, cardiac, bone and neural tissue engineering applications and stem cell differentiation. Thus cryogel polymeric biomaterials have shown promising applications in tissue engineering and regenerative medicine and this talk will give an overview on these potential areas.

Key words: Cryogel monoliths, Cryogel bioreactor, Cell separation, Bioartificial lever, Skin, Cartilage and Bone tissue engineering.
Titanium alloys are becoming more and more popular for medical applications as components of joint implants due to low weight, good mechanical properties and excellent corrosion resistance. The emphasis of the latest research is on improving the biocompatibility of titanium alloys by surface modification. Therefore the hydroxyapatite (HAp) coating was deposited on two phase (α+β) Ti-6Al-7Nb titanium alloy by micro-arc oxidation (MAO), process in electrolyte containing (CH3COO)2CaH2O and Na3PO4. Microstructure of the coating was investigated using scanning electron microscopy (SEM), transmission electron microscopy (TEM) and X-ray diffractometry (XRD). The coating was composed of nanocrystalline HAp crystals (hexagonal close-packed; hcp) with needle-like shape. The coating exhibited high porosity and very high surface development (Fig. 1). The open pores with diameter up to 1 μm and complex geometry as well as closed ones with diameter up to 500 nm were observed. Porous surface allows for cells ingrowth thus facilitating bone/implant integration. To investigate the biocompatibility of obtained composite material preliminary in vitro studies were done. MTS assay, DNA quantification, ALP activity and confocal microscopy observation were performed after one day culture of mesenchymal stem cells on coated samples and control samples (uncoated Ti-6Al-7Nb alloy). Preliminary results show no significant difference in cells adhesion, proliferation and enzyme activity between analyzed samples. Further, more complex biocompatibility analysis are in progress.

Fig. 1 . SEM micrograph of the HAp coating deposited on Ti-6Al-7Nb alloy by MAO
Innovative Biodegradable Synthetic Scaffolds for Vascularized Bone Tissue Engineering

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Hydroxyapatite (HA)/polymer composites have been studied as alternative graft material for the treatment of large bone defects. In this study, polyurethane foams PUFs were studied in different compositions: Poly(ether urethane urea) PEUtU, poly(ether urethane) cross linked by polysaccharides PEtU and poly(ester ether urethane) PEEtU. PUFs has been filled by HA in order to increase toughness, mechanical properties and bioactivity. Our innovative technique, PCT patented, to grow 3D channelization inside porous bulk was applied to improve fluidics and angiogenesis. Mechanical properties and in vitro cellular response were investigated. The scaffolds were implanted in mice and in vivo bioactivity was evaluated by µCT, in vivo imaging and histological sections. PUEt improved the mechanical properties, potentially suitable for weight-bearing applications. All composites are biocompatible and supporting differentiation of human osteoblastic cell line, with PUEEt enhancing the cell viability inside the core scaffold. Implanted scaffolds were biocompatible, with PUEEt even without adverse inflammatory response. Additionally, there is evidence of cellular infiltration in pore and bulk, adhesion of cells to pores wall and initial extracellular matrix deposition. Moreover, channelized scaffold greatly support angiogenesis. Our results suggest that those innovative PUF compositions could be potentially useful biomaterials for bone TE, above all for large defects satisfying vascular need.
Growth and Potential Damage of Human Bone-Derived Cells on Fresh and Aged C60/Ti Composites

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Thin films of the binary C60/Ti composites, with various concentrations of Ti ranging from ~25 % (i.e., 25 Ti atoms and 75 C60 molecules) to ~70 %, were deposited on microscopic glass coverslips in a micro-patterned form through a metallic mask, and were tested for their potential use in bone tissue engineering. Numerous studies have evaluated the therapeutic potential of fullerene derivates against oxidative stress-associated conditions, including the prevention or treatment of arthritis. On the other hand, fullerenes and their derivatives are not only able to quench but also to generate harmful reactive oxygen species, which could lead to the inhibition of cell growth, cytotoxic injury or cell death. The reactivity of C60/Ti composites may change in time due to the oxidation and polymerization of fullerenes in an air atmosphere. In this study, we therefore tested the dependence between the age of the C60/Ti films (from one week to one year) and the proliferation, viability and metabolic activity of human osteosarcoma cells (lines MG-63 and U-2 OS). We also monitored potential membrane and DNA damage as well as morphological changes of the cells. After 7 days of cultivation, we did not observe any cytotoxic morphological changes, such as enlarged cells or cytosolic vacuole formation. Furthermore there was no increased level of membrane or DNA damage. Moreover, there was no effect of the age of the C60/Ti layers on the viability, cell population densities or on the DNA damage response. Therefore C60/Ti composites could be considered as a promising material with potential use as a bioactive coating of cell carriers for bone tissue engineering.
Tissue engineering studies use cells that were taken away from their natural environments. We hypothesized that autophagy activation might be an important survival response against stress faced by cells under these conditions, and this might affect cellularization on man-made scaffolds. Autophagy is a major stress response against hypoxia, nutrient limitation and detachment from substrate, conditions that are also observed in tissue engineering studies. This biological process allows the cell to recycle long-lived proteins and damaged organelles. Following sequestration in double or multimembrane autophagic vesicles, cellular cargo is delivered to lysosomes for degradation. Thus, autophagy plays an important role in the elimination of abnormal or misfolded proteins and damaged organelles, sustains intracellular building block-nutrient levels using cells’ own resources, so, allows cells to survive during stressful periods. Here, we analyzed the importance of autophagy in growth, survival, attachment to substrate and migration of cells. Moreover, we also tested the importance of the optimization procedures during cell-based bio-ink preparations. Our results showed that, stress-activating conditions (nutrient and serum starvation, hypoxia and incubation time in printing capillary) led to autophagic responses and affected cell survival. In line with this, in autophagy-deficient cells, death by apoptosis was potentiated during the bioprinting process. All these results introduce autophagy as a novel and important parameter for tissue engineering studies and bio-ink cell survival during tissue printing.

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Skin is the largest organ of the body acting as a first defense against infection, temperature changes and damage to internal organs. When the skin is extensively damaged and needs to be reconstructed, the golden standard is transplantation of split-thickness skin, composed of the epidermis with a small part of the dermis. However, lack of dermal tissue results in scarring and wound contraction. To improve the clinical outcome, novel cellular skin substitutes have been developed by the EuroSkinGraft Consortium. Generally, the evaluation of skin substitutes for quality control purposes is based on qualitative parameters, such as histology, rather than quantitative parameters. Quantitative analyses, however, allow for a meaningful comparison of skin substitutes between clinical and/or research centers, are unbiased and less prone to observer-specific interpretation. This study focuses on the identification of markers that reflect the quality of skin substitutes and may provide the basis of objective evaluation and quality control of skin substitutes. We focused on biochemical and molecular biological analyses. In this study, a dermal and a dermo-epidermal skin substitute were investigated. Using qPCR, the gene expression profile for specific dermal and epidermal genes was analyzed. Of all genes analysed in the dermal construct, elastin mRNA expression was most prominent indicating the active regeneration of the ECM by the fibroblasts. Epidermal gene expression gave a good indication on the differentiation state of the dermo-epidermal skin substitute and was, as expected, absent in the dermal construct. For quantitative analysis at the protein level, extracellular matrix molecules were determined using ELISA assays, but poor extraction of these proteins compromised accurate measurements. As an alternative enzyme activities were evaluated, representing a functional read-out parameter. For the dermal part of the skin substitute, the activity of lysyl oxidase, an enzyme responsible for crosslinking collagens and elastin in the extracellular matrix, represents a good candidate.

The (dis)-advantages of biochemical and gene expression data for the evaluation of skin substitutes will be discussed.

Keywords: Skin substitute, keratinocyte, fibroblast, quality control

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Intra-synovial tendon injuries display poor healing. Autologous bone marrow mesenchymal stem cells (BM-MSCs) can enhance tissue repair in tendon injuries, however their distribution, and resulting function, in an intra-synovial environment is unclear. Synovial membrane (SM) has resident MSCs displaying similar characteristics to BM-MSCs. This study used a tenoscopically created sheep model of intra-synovial tendon injury of the deep digital flexor tendon (DDFT). BM- and SM-MSCs labeled with Molday-ion iron-oxide nanoparticles (MIONs) were injected into the digital flexor tendon sheath (DFTS) of the forelimb to track their distribution by MRI.

Multipotent stromal cells were isolated from sheep BM and SM biopsies. Cells were labeled with MIONs (BioPal, USA) and injected into the ovine forelimb DFTS. The lateral DDFT had a longitudinal defect created 7 days earlier. Cells were tracked in the forelimbs at 7 days post-injection with a 1.5T MRI scanner (Philips). Control sheep had PBS injections. MRI scans showed that both MSC types were distributed throughout the DFTS. Distribution was uneven and cells adhered to the synovial membrane. Cells that may have adhered to the tendon defect could not be detected easily due to poor contrast. PBS treated and contralateral forelimbs (no surgery or cell implantation) lacked MIONs-related signal.

The study demonstrates evaluation of parameters for injection of MSCs into the ovine DFTS, and cell tracking by MRI. It proposes MION labeling to be safe and useful for cell tracking in vivo. MSC distribution after intra-thecal administration doesn’t seem to be generalized. Some cells may adhere to the tendon defect, the majority to the synovium.
The Effect of Bone Marrow Derived Mesenchymal Stem Cells on Repair of Surgically Induced Core Lesions of the Equine Superficial Digital Flexor Tendon

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High prevalence of superficial digital flexor tendon (SDFT) injury is an major cause of wastage in performance horses. We hypothesized that injection of core lesions with BM-MSCs suspended in bone marrow (BM) supernatant, would result in repair tissue more similar to normal tendon than injection with BM supernatant alone. Standardized core lesions were created in the SDFT of both forelimbs of 6 horses. Ten x 106 cultured autologous BM derived mesenchymal stem cells (BM-MSCs) were injected intra lesionally. Control limbs were injected with 2 ml citrated BM supernatant. After 1 week rest, horses resumed an ascending exercise regime. Horses were euthanized 12 weeks after implantation of MSCs. Serial ultrasonographic examinations were performed until 12 weeks post injection. A 1.5T MRI evaluation was performed immediately after death. Tendon sections from the maximum injury zone were evaluated histologically. Tendon segments from the maximum injury zone were also prepared for electron microscopy. The Mass Average Diameter, the Collagen Fibrillar Index, and the Area Dependent Diameter values were calculated from electron micrographs of treated and control normal regions, and treated and control injured regions. No significant differences existed between treated and control limbs with respect to ultrasonographic parameters, MRI measurements, histologic assessments and electronmicroscopic evaluations (MAD, CFI, and ADD values).

Repair in 16-week-old tendon core lesions was not found to be different in limbs treated with BM-MSCs suspended in BM supernatant, when compared to control limbs, using ultrasonography, magnetic resonance imaging, histology or electron microscopy.
Immunophenotype and Gene Expression Profiles of Cell Surface Markers of Mesenchymal Stem Cells Derived from Equine Bone Marrow and Adipose Tissue

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Horses are both patients and preclinical models for human musculoskeletal disorders. Mesenchymal stem cells (MSC) are able to self-renewing and differentiate to mesoderm tissues, which has become them in the main stem cell therapy that is applied to musculoskeletal injuries. Bone marrow and adipose tissue are the two main sources of MSC and, despite of the fact their characteristics are well known, there is not at unique surface marker to identify them. In addition, commercial antibodies commonly used in flow cytometry to sort MSCs are designed against human or mouse epitopes, which complicates the characterisation of the surface markers of equine MSCs. The aim of this work was to analyse the immunophenotype of 7 surface markers and the expression of a panel of 13 genes coding for cell surface markers in equine bone marrow and adipose tissue-derived MSCs from 9 horses. The tri-lineage differentiation was confirmed by specific staining. Equine MSCs from both sources were positive for the MSC markers CD29 and CD90, while they were negative for CD44, CD73, CD105, CD45 and CD34. The gene expression of these molecules was also evaluated by reverse transcriptase real-time quantitative PCR along with the expression of 6 other MSC markers. Both populations of cells expressed CD13, CD29, CD44, CD49d, CD73, CD90, CD105, CD106, CD146 and CD166 transcripts. Significant differences in gene expression levels between BM- and ATMSCs were observed for CD44, CD90, CD29 and CD34. Both cell types were negative for CD45 and CD31. The complementarity of both techniques allowed to establish the surface marker pattern of MSCs and revealed a similar phenotypic profile between horse and human MSCs, although specific differences in some surface antigens were noticed.

Keywords: Horse, MSC, cell surface markers, immunophenotype, gene expression
Resources, transaction costs, internalization, organizational learning, and real options all play a role in the decisions made by companies to acquire or partner an external asset. Empirical evidences suggest that licensing deals remain the preferred partnering mode for Big Pharma to access an external asset, as this transaction mode limits the risks taken and leaves operational costs in the hands of the Biotech partner and its financial backers. However, several factors may tip the balance in favor of M&As; these include: (1) business factors such as the need to access contracts that are not assignable or the desire to limit transaction costs with a potentially long term partner, and (2) competitiveness factors such as intellectual property that is not accessible otherwise or that represents a critical success factor, or know-how that is difficult to transfer. Considering the absence of convincing therapeutic stem cell products on the market, the perceived technology and business risks remain high for regenerative medicine despite the successes of Dermagraft and Apligraf, suggesting that, for investors, the welcome exit via an M&A is an elusive possibility in the short term. This results in the absence of pressure for Big Pharma to compete for regenerative medicine assets though conventional licensing.

What thus is the best answer? Backed by robust science fundamentals, perhaps a focus on developing even a single successful stem cell product for any indication would be sufficient to trigger a surge of interest by Big Pharma, as long as the clinical benefits delivered are transformational, i.e. that only a stem cell product can deliver. The proposal here is that a rare disease unequivocally remediated by a product from the new therapeutic modality class would be sufficient to demonstrate that the concept of regenerative medicine is ripe for industrialization, thus enabling large technology deals. This would benefit the early Big Pharma adopters by internalizing critical success factors to replenish product portfolios with a range of novel franchises, where barriers to entry would be constituted not only by the freedom to operate, but also by R&D and manufacturing know-how and trade secrets. Furthermore, Biotech companies would reap non-cash benefits such as deep disease biology knowledge, regulatory, manufacturing, as well as marketing competences and resources. Probably even more so than for partnerships involving conventional products, robust alliance management processes here constitute a key capability to move forward through the R&D life cycle and the translational process, as it involves multiple stakeholders and functions including translational centers, cGMP manufacturing, and clinical development.
Human Placental Extracellular Matrix Based Medical Devices

W. Hofgartner

Celgene Cellular Therapeutics, Warren, New Jersey, USA

Celgene Cellular Therapeutics (CCT), a wholly-owned subsidiary of Celgene Corporation, discovers and develops therapeutics from cells and biomaterials derived from the human placenta. CCT, based in Warren, New Jersey, is a state-of-the-art research and development organization with in-house GMP manufacturing, dedicated to developing cutting-edge products and therapies. CCT has developed cost-efficient technology to extract human placental extracellular matrix (ECM), which is a versatile and engineerable biomaterial that can be formulated and configured into a variety of product embodiments. ECM-based medical devices offer near term commercial opportunities in a number of therapeutic areas.
Developing Flexible Cell-Therapy Processes to Balance Clinical Trial Needs with Future Commercial Operation

Paul Kemp
Intercytex Ltd Core Technology Facility, Manchester, UK

A company developing a new cell therapy has to make several key decisions very early in the development process relating to operations and manufacturing that will have a long term impact on the product's eventual commercial viability. These decisions are impacted by a variety of scientific, regulatory and financial considerations and often compromises need to be made in order to satisfy all stake-holders, particularly in the early stages when funding is rate limiting. Intercytex has many years' experience in developing cell therapies and has carried out a number of clinical studies in the U.S. and EU with both autologous and allogeneic products. It is currently developing an injectable, allogeneic cell therapy for the treatment of a number of skin conditions as well as making its expertise and GMP manufacturing capability available to others through its Cell2Therapy service. This talk will use real examples to illustrate the kinds of decisions that need to be made and when during the translation of a therapy from the bench, to the clinic and then the market and the potential pitfalls and risks inherent in the decision making process.
Gelatin-hydroxyapatite Cryogels for the Regeneration of Critical Sized Calvarial Defect in Rat Model

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In recent years, several tissue engineering techniques were used to improve bone healing in critical sized calvarial defects. In this study gelatin-hydroxyapatite cryogel scaffolds were used with/without bone morphogenetic protein-2 (BMP-2) and transforming growth factor beta-1 (TGF-β1) to heal critical sized bone defects. Biocompatibility of prepared cryogel scaffolds were analyzed by MTT, alkaline phosphatase assay, scanning electron microscopy (SEM) and confocal imaging analysis. In vivo study groups were examined by micro computerized tomography (μ-CT) and histology on 2 months and 4 months after surgery. Gene expression studies were performed on 2 weeks and 4 weeks after surgery. Collagen type I (Col I), alkaline phosphatase (ALP) and osteocalcin (OSC) gene expression levels were analyzed. Cell viability of gelatin-hydroxyapatite cryogel was 92.77±0.06 % in MTT. SEM and confocal imaging analysis showed excellent attachment and migration of cells in the scaffolds. In ALP assay, relative ALP activity of gelatin-HA cryogel was 363.14±5.96 % but relative ALP activity of gelatin cryogel (without HA) was 179.32±11.20 % on 14th day. Histology and μ-CT analysis showed there was significant bone healing in the group which includes BMP-2 and gelatin-hydroxyapatite scaffold (Group I) as compared to other groups except autograft applied group (Group IV). Significant increases were observed for Col I, ALP and OSC in Group I and Group IV. However there was no significant increase in the group which had BMP-2 and TGF-β1 applied together (Group II). The results reveal that combination of gelatin-hydroxyapatite scaffold and BMP-2 is a promising candidate for bone healing in critical sized craniofacial bone defects.

Keywords: gelatin; hydroxyapatite; cryogel; bone tissue engineering; bone morphogenetic protein-2; transforming growth factor beta-1
AgNPs and Materials Carrying them: Antibacterial But May Be Cytotoxic

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The emergence of bacterial resistance to antibiotics following widespread clinical and veterinary usage has made antibiotics much less effective means that we are now facing the threat of these very dangerous/powerful enemies - pathogenic bacteria - resistant to most or all available antibiotics. Thus, exploring alternative approaches to develop antibacterial agents is an urgent task and getting a tremendous attraction as expected/needed. Silver has been in use since ancient times in the form of metallic silver, silver nitrate, silver sulfadiazine, etc. to make water potable initially and then many years for the treatment of burns, wounds and several bacterial infections. They have been replaced by antibiotics in time, however – recently it seems that the interest have returned back to silver - especially in nanoparticle form mainly due to their high surface area to volume ratio and also other unique chemical and physical properties. Silver ions are highly reactive on almost all kinds of bacteria, there are different scenario about their action, mostly agreed that they can bind to proteins both on the bacterial wall and and incytoplasm and cause denaturation which brings structural changes leading to cell distortion and death. They can bind to bacterial ribosomes, genomic DNA, RNAs and several proteins which inhibits bacterial replication. AgNPs may also interact with proteins on bacterial wall and may even enter the bacterial cell in their free forms (nanoemulsions). They preferably do attack the respiratory chain, cell divisonfinally leading to cell death. However, most likely silver ions released from their surfaces, determines the bactericidal activity when they are immobilized or entrapped on/in carriers matrices (like fabrics, gels, etc.). Antibacterial properties of both AgNPs and their immobilized forms on a series of bacteria (mainly E.coli and S.aureus, but also others) have been investigated by several groups. There are similar but also controversial reports, which is understandable because both the materials and the experimental (test) conditions are quite different. For instance type of bacteria and/or their species; inoculation densities of bacteria (relative to the amount of AgNPs used); AgNPs type, size/shape, the material properties carrying them; incubation period, temperature, pH and ionic strength are all different, therefore most of the time these results are not even comparable. One of the important points in these studies - worth to note- is the design of these studies which have mostly attempted to demonstrate the antibacterial performances of their systems -both the AgNPs and materials carrying them. Silver almost in all forms is toxic on mammalian cells. The cytotoxicity both human, animals, and of course on environments should be very critical issue. This presentation overviews two critical issues, “antibacterial vs cytotoxicity” by using related literature data and our studies.
Safety and Performances of ATMP Before First in Man Studies

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This lecture will address some toxicologic, scientific and regulatory requirements before use of ATMP during first in man studies.

• The basic principles for risk analysis will be introduced and specific aspects of the existing regulatory requirements will be exposed
• When applicable the use of literature, in vitro and in vivo safety preclinical studies will be presented
• The relevance and application of performance evaluation at the non clinical stage will be discussed
• Practical examples will be used to illustrate some principles for safety and performance evaluations of ATMP prior to clinical uses. Appropriate and relevant design of a clinical trial is critical to ensure that it will generate expected data regarding drug toxicity, drug efficacy and appropriate risk/benefit balance for treated patients.
Appropriate and relevant design of a clinical trial is critical to ensure that it will generate expected data regarding drug toxicity, drug efficacy and appropriate risk/benefit balance for treated patients. But, since clinical observations provide only partial information, where data interpretation is complex and affected with significant statistical variations, additional data have to be generated to improve the characterization of the biological activity and the understanding of the product’s mechanism of action. For numerous indications such as cancer, infectious diseases, chronic inflammations or auto-immune diseases, immuno-modulating therapeutic strategies have to be evaluated through appropriate immunomonitoring studies. Carefully designed and carried-out clinical (or preclinical) immunomonitoring studies provide key information on the immune response of a patient to a treatment. These data can be correlated with clinical observations, corroborate a hypothetical mechanism of action and consolidate regulatory packages to support product registration and/or next clinical development steps. In particular with Advanced Therapeutic Medicinal Products, such as cell based therapies, the product characterization and the measurement of its biological activity are crucial. Indeed, complexity of cell-to-cell interaction adds to the complexity of immune responses. Thus any data deciphering this intricacy and providing visibility on the drug mechanism of action will be a strong asset for the drug development, especially to go through its regulatory path.

Numerous technologies have recently been developed to improve characterization of the immune response, increasing sensitivity, accuracy as well as the number of parameters simultaneously measured. But a strong know-how in immunology and bio-assay validation is required to obtain, from such technological platforms, reliable data. These tools are also very useful for the development of biomarkers to be validated in parallel of the therapeutic product.
The commercial opportunities for new medical devices are often tied to their value to healthcare providers. This means that the costs of developing and producing a new device must be covered at a price consistent with reimbursement norms. With this in view, it is desirable to estimate a value-based price ceiling at an early stage of the development cycle and to keep the estimate under continuous review as more evidence accumulates. We outline a framework in which the estimated price ceiling or “Headroom” is used (a) as a ‘reality check’ on the viability of the device in the healthcare market, (b) to support development decisions and (c) to contribute to a pricing policy which respects uncertainties in the reimbursement outlook. Early-stage decision-making is hampered by limitations in the available evidence-base, but useful headroom estimates can often be obtained. Examples will be drawn from among recent new medical products.

The proposal reflects an evolving attitude to uncertainty over the course of the development cycle, based on the timing and manner in which new information accrues. Within this framework the developmental value of new information can justify the costs of clinical trials and other evidence-gathering activities.
Cellular biology drives the field of regenerative medicine and its continuous evolution allows for the development of manufacturing processes and operations that generate safer and more efficacious products. The understanding of cell plasticity and interactions with the environment are two of the key factors for the development of in-process controls and final cellular product specifications. In the ATMP world it is important to keep in mind that the product consists of cells and milieu which interact in complex and dynamic ways.

In the late 80s the primary goal of tissue engineering was to build complex tissues in laboratory settings. In the field of skin biology research was focused on the development of skin “equivalents” and gave rise to products termed “skin substitutes”. Cells were cultured to fabricate the tissue in vitro. This generated long, tedious and costly processes. Since that time the combined knowledge of wound healing science and regenerative medicine has resulted in new perspectives. The current goal is not to induce cells to make a product but rather to produce products that better utilize the intrinsic potential unique to each cell type. It is in harnessing this potential that a shift in thinking from replacing tissue to helping the patient’s own tissue heal itself has occurred. This shift in concept from tissue engineering to single cell suspension application has resulted in the development of new technologies with less cumbersome manufacturing processes and improved efficacy.

Several questions arise when entering the ATMP world including questions about the planning of operations for the development of new ATMPs and how to deal with the various challenges encountered along the way. Such challenges include manufacturing development, in process control, clinical design, product development, process development, and facility and equipment design, all of which are substantially different from those applied in typical drug development.

When performing process development and scale up of cellular therapies at least three main drivers should be always taking into account: (1) materials and reagents involved in the production process must be well defined, (2) cell manipulations performed during manufacturing should be kept to a minimum and, (3) specifications of the final product must be broad enough to tolerate the biological variability of normal living organisms without compromising the batch to batch similarities.

Finally, production of quality cell-based products and their proper application requires unique skills from the R&D scientists to clinicians in order to improve the quality of life of the patients we serve.
A Risk Based, unit Operation Approach to Manufacturing Process Science for Cell Based Therapies’

Robert Thomas

Loughborough University & EPSRC Centre for Regenerative Medicine, Loughborough, UK

The EPSRC Centre for Innovative Manufacturing in Regenerative Medicine is focused on bridging the manufacturing science gaps necessary for industrial production of advanced therapies. A key goal is to reduce the risk and cost of translating development stage advanced therapies to commercially viable products. An important step is early process analysis, consideration of constituent units of operation, and risk assessment in the context of industrial operating restrictions and logistics. This requires an understanding of the tolerance of the development process critical outputs to industrial manufacturing relevant process parameters to inform a risk:benefit assessment of any specific production strategy; this is particularly challenging for cell based therapies, in part due to deficiencies in understanding of the relationships between measured cell attributes and functional process or product performance. Key challenges such as identifying units of operation, required measurement system performance and frequency for process control, and application of engineering design for optimisation, risk reduction, and decision making, will be discussed with examples from both academic and commercial cell-based process development programmes on-going in our laboratories.
The need for organ transplants is currently exceeding the supply of donors from the general public. To this end researchers have been exploring Bio artificial replacement models to be used as substitutes for organ replacements. In order to generate representative numbers of cells to mimic the activity of a complete or partial organ, the manufacturing capacity of appropriate cells has to be scaled up, especially because of the fact that the cells needed for the generation of these devices require adherent cells. Current established manufacturing methods and technologies for adherent cell expansion are limited to approximately 100 billion cells per run from an operational perspective and a cost perspective. As the cell therapy and regenerative medicine develops and matures in clinical phases, the industry has developed multiple scalable platforms which could be utilized for >500 billion cells per run. Here we would like to discuss a case study regarding cost effective the development of such a platform for adherent cell culture through strategic partnerships.
Enabling Allogeneic Cell Based Product Manufacturing Transition from R&D to Industrialization
Case Study by Promethera Biosciences - A Cell Therapy Company

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Promethera Biosciences® is a Belgian Biotech company, producing the cell therapy product, HepaStem, to treat serious metabolic liver disorders. This treatment is based on human heterologous liver progenitor cells isolated from healthy adult livers. A European phase I/II clinical trial is currently ongoing for the treatment of Crigler-Najjar syndrome and Urea Cycle Disorders in a pediatric setting. To further upscale the process, minimize manual operations & related-risks, as well as to reduce overall costs, Promethera has developed a fully closed system. Currently, the manufacturing process is performed in open aseptic conditions using CellBind-treated CellStacks. The next generation production process will be based on ATMI’s Xpansion 2D multiplate bioreactor technology. This multiplate bioreactor offers a similar surface treatment & area, allowing for an easy transfer from the existing multi-tray stacks process. But in addition, it offers a fully controlled environment via real-time monitoring of temperature, dissolved oxygen, pH and media flow rate, reducing in-process variations. Monitoring of cell morphology is accomplished via Ovizio’s digital holographic microscopy.
To date, Promethera’s liver progenitors were successively expanded in XP10 (6120 cm2), XP50 (30600 cm2), and XP180 (110160 cm2) without change in growth rate, cumulative population doubling time, homogeneous distribution, and cell quality (identity/purity and potency). A substantial increase in cell yield was achieved.
The presentation will address the manufacturing challenges faced by Promethera when transitioning from Research & Development to pilot and industrial production process. The strategy to overcome these challenges will be presented with focuses on equipment design and validation, operational development costs as well as future perspectives.

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Practical Advice on How to Move Translational Process Forward

Y Bayon, S Ellison, John Barry, Rui Amandi da Sousa, Vincent Ronfard, Paul Stroemer

TERMIS-EU Industry Committee

The 4th edition of the TERMIS-EU Industry Symposium will give practical advice on how to successfully translate regenerative medicine into commercial solutions: plan realistic development milestones to take development pipelines forward; utilise sometimes hard, often complex decisions to advance their translational initiatives into the development of commercially viable solutions.

The TERMIS-EU 2013 Industry Symposium aims to build on an introductory lecture, highlighting the business perspective of the translational process of regenerative medicine solutions, by focusing on three topics:

- Translational Centres - How centres can help individuals move the industry forward
  Highlighting the relationships/partnerships between stakeholders, failures and successes in promoting and developing regenerative medicine solutions, and the future for successfully translating regenerative medicines.

- Operations Management - How to move a process into the GMP environment
  To give, from real industrial experiences, answers to key questions related to operations planning, the manufacturing facility & equipment design, the qualifications & validations of the manufacturing equipment and plant, and operational budget management.

- Clinical Translation - Moving a GMP process into robust trials
  To address key topics of clinical strategy, planning and management of regenerative medicine solutions, and illustrate decision tools based on health economics & market analysis (eg. QALY and cost-effectiveness analyses).
Using the UK infrastructure to Overcome Translational Boundaries

Simon Ellison

Cell UK Catapult & NHS Blood and Transplant, Oxford, UK

Completion of PhIII trials and/or being able to treat patients on a wide scale is the primary aim cell therapy organisations. However the, well known, translational challenges such as sourcing funds, regulatory expertise and manufacturing often impede efficient progress. The UK has a unique infrastructure built around the manufacturing and logistics of NHS Blood & Transplant, and the regulatory, clinical and funding understanding of the Cell Therapy Catapult, gives developers. NHS Blood & Transplant has a validated capability to consent, manufacture and deliver cell based therapies. This infrastructure has been built on its management of the entire stem cell, blood, tissue and organ value chains. This national skill set is now available to cell therapy developers to assist in translation. The Cell Therapy Catapult is a translational hub established by the UK Government with the capability and experience to assist any organisation overcome any translational barrier. The Catapult operates with therapies that have left research but not reached PhIII, and operates as a collaborator by inputting experience and knowledge to enable companies to efficiently treat patients. The barriers to commercialisation are well known but how to overcome them is subject to much debate. This presentation will illustrate how therapy developers can use the existing skills and expertise within the UK to accelerate through clinical trials and into market authorisation. This will be achieved by illustrating the existing skills in the UK and showing case studies of how they can be immediately used to collaboratively move therapies forward.

The Andalusian Initiative for Advanced Therapies -a publicly funded organization created in 2008 by the Andalusian Government, Spain- was established to promote the development of new therapies with the view to improving the health of the population, driving innovation in our health care system and encouraging progress for our Region. To do this, we seek alliances with the academic world, research institutions, health centres, patients' associations, SMEs and the pharmaceutical industry. Over the last few years, in collaboration with different Universities and Pharmaceutical companies, we have pushed forward the building of a cluster of research centres and institutes, biobanks and Good Manufacturing Practice (GMP) facilities. The Andalusian Initiative offers a distinct, major translational focus. Thus, our organization provides a comprehensive support hub to develop clinical research. Support comes not only in the form of financing, but also with implementation of complementary expertise (we have also designed, in collaboration with the University of Granada, a European Master Degree in Manufacturing of Advanced Therapies Medicinal Products).

To summarize, we have built a network of 10 GMP facilities in multiple research centres, tissue banks and hospitals thus far. We currently support their accreditation and the development of investigational medicinal products, and the design and implementation of clinical trials. In the case of the latter, our support begins with a regulatory assessment during preclinical development, followed by the promotion of collaborations between basic and clinical researchers, and extended up to monitoring the safety of the resulting investigational medicinal products. As a result of this organizational system, we have already acted as sponsors of 18 Phase I/II clinical trials in the areas of cardiology, neurology, immunology, peripheral vascular disease and hepatic regeneration among others. At present we are collaborating with some biotechnological companies and other academic institutions in order to further development of these and other investigational medicinal products.
Advancing a new technology concept to the market is an enormous undertaking. Bundling several well-aligned products together into a sustainable company, especially in the life sciences, is fraught with hurdles and risk. Bringing emerging companies, academic institutions, established companies, investors and government together to build an industry—that is near impossible. Yet, that is the challenge and opportunity embodied in a global network of support organizations emerging around the world. These so-called translation centers are typically public-private partnerships tasked with a mission to bridge the “valley of death” in the commercialization pathway. The proliferation of these centers in recent years is a direct response to a historically poor return on investment by government and investors in biotechnology and the resulting capital-limited environment for new opportunities like regenerative medicine. The proliferation of these centers has generated new models of collaboration, frameworks for industry-academic partnerships, policies for enabling intellectual property sharing and vehicles for financing opportunities. It has also stimulated the formation of consortia of translation centers—a further indication of the great level of cooperation needed to advance an emerging science to the patient bedside. The Centre for Commercialization of Regenerative, a two-year-old translation center based in Toronto, Canada, and its network of government, industry and academic stakeholders, will be used to present the critical characteristics, policies, and activities of an RM-focused translation. In particular, the presentation will highlight the Canadian focus on building multi-disciplinary “ecosystems”, i.e., collaborative networks, to support commercialization. Early successes, “lesions” learned and future potential models of translation will be discussed.
Antibacterial Effect of Electrospun PCL Membranes Loaded with Silver Nanoparticles

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In the study, antibacterial effect of electrospun poly(ε-caprolactone)(PCL) membranes loaded with silver nanoparticles has been investigated for possible biomedical applications such as wound dressing. Electrospun membranes of PCL was loaded with different amounts of Silver nanoparticles. Loading of silver nanoparticles inside the swollen membranes was done by immersion of these membranes inside the suspension of silver nanoparticles with different concentrations. In order to determine the minimum effective loading concentration, disks (6 mm in diameter) loaded with silver nanoparticles were tested with *Staphylococcus aureus* (gram positive) “Disk Diffusion Assay”. The anti-biogram disk diameters were measured and reported as a measure of antibacterial activity and compared with a nonwoven fabric which was used as a positive control. The anti-biograms for silver nanoparticles loaded PCL membranes showed antibacterial effect compared to control and anti-biogram disk diameter decreased with decreasing amount of silver nanoparticles loaded onto the nonwoven disks.
Selection of Bacteriophages for Different Practical Applications

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Bacteriophage (from 'bacteria' and Greek φαγεῖν phagein "to devour") is any one of a number of viruses that infect bacteria. Bacteriophages (shortly "phages") are among the most common and diverse entities in the biosphere. The estimated number of phages on earth is about $10^{31}$. Sea waters, human intestines, etc. are natural sources of bacteriophages. Phages are not only the most abundant biological entities but also probably also the most diverse ones.

Bacteriophage mixtures ("cocktails") have been traditionally used for therapy and prophylaxis of bacterial infections in Georgia. The phages nowadays are seen as a possible therapy against multi-drug-resistant strains of many bacteria.

The phages aimed for therapeutic or prophylactic applications usually are isolated from the sewage collectors, rivers, lakes and ponds. Study of biological properties is the main criteria for proper selection of bacteriophages. For therapeutic and prophylactic phages this is essential to prove their virulent nature, which is based on their plaque and virion morphology, broad host range, serological and genetic relatedness, etc. Special requirement are drawn out for diagnostic and/or marker phages (e.g. narrow host range, survival of bacteriophages in the certain environmental conditions such as pH, UV irradiation, etc.) used for environmental modeling. The phages aimed to be used for veterinary purposes should be isolated from the farm environment and be adapted towards the farm isolates. In this case the phages may be used for therapy of prophylaxis, diagnostic or sanitary purposes for reduction of the number of pathogenic bacteria or their complete eradication. Phage preparations may be used for prophylactic treatment of plants as well. Accomplishing of the above mentioned tests helps to differentiate the phages applicable for various practical purposes.

The efficacy of phage preparations largely depends on a manufacturing process enabling phages to be obtained in a high titer, etc. The elaboration and development of manufacturing regulations and methods may be considered as one of the most significant achievements of the Soviet scientists.

The presentation will focus on such important issues as the safety of the phage preparations, quality control methods, etc. The commercial phage preparations and their practical applications will be discussed as well.
Hyperthermia with RF Using Nanoparticles

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Although a lot of progress have been achieved in the diagnosis and treatment of cancer, their current levels are not fully satisfying (Greenlee, Hill Harmon et al. 2009). Classical cancer therapy techniques include surgery, chemotherapy and radiotherapy have been limited due to their side effects. Hyperthermic techniques include metallic base nanoparticles and radiofrequency (RF) induction have made new insights into their use in biomedical diagnostic and therapeutic applications of cancer treatment and gain a great importance due to lower unwanted harmful effects. In standard hyperthermia, an increase in body temperature of up to 5°C above normal level leads to the death of cancerous cells via "tumor ablation".

In this study, the effects of RF power exposure on the different kinds of nanoparticles (shape, type and size) have applied and hyperthermic temperature raise have been investigated. Supermagnetic iron oxide nanoparticles (SPIONs) has synthesized by co-precipitation method, Au@SPIONs, AuNRs and AuNPs have synthesized by certain methods. An antenna module RF generator with 144.000 MHz and 200 W RF power have been designed, produced and used as an energy source. Nanoparticles alone experiments (with RF) intended to temperature raise observations into the RF combination. MCF-7 (Breast cancer) and L-929 (Fibroblast) cell lines have been cultured for the later stage in order to use in in-vitro experiments.

During experimental steps, temperature increased values, caused of RF power exposure on diverse nanoparticles conditions (shapes and concentrations) have followed by infra-red thermal camera images. Cell death effects of hyperthermic heat have been studied on L-929 (Fibroblast) and MCF-7 (Breast cancer) cell lines during in-vitro experiments. In preliminary experiments, various RF powers have been induced (80, 120 and 180w) on different nanoparticle dispersion (5, 15, 30ppm). Temperature increasing values have observed by infra-red thermal camera and have been recorded. The same conditions has applied on the L-929 and MCF-7 cell lines in in-vitro experiments. Viabile, apoptotic and necrotic cell death indexes have evaluated for both cell lines. According to the results, it was observed that the highest temperature, 44 °C, has obtained by applying highest RF power exposure on SPIONs with the maximum nanoparticle concentration (30 ppm). In conclusion, refer to above conditions (180W RF power, 30ppm SPIONs Conc.) the highest apoptotic cell death value (62±7%) has observed at MCF-7 cell lines (in-vitro experiment).
“Adipose-derived Stem Cells Biocompatibility and Chondrogenic Potential Assessment in Novel Chitosan-β Glycerophosphate Scaffolds Designed for Cartilage Reconstruction”

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Natural compounds are currently required for the improvement of tissue engineering strategies. This study aims to assess the biocompatibility and the chondrogenic differentiation potential of human adipose-derived stem cells (hADSCs) in contact with a novel chitosan-βglycerophosphate 3D matrix (CHTβGP) designed for cartilage regeneration. hADSCs in the 4th passage were seeded on CHT surface but cells managed to populate deeper layers of the scaffold, resulting in a 3D culture. This system was evaluated in terms of biocompatibility by quantitative and qualitative assays. Furthermore, the bioconstruct was exposed to chondrogenic induction cocktail for 28 days and the process was monitored after 7, 14 and 21 days of differentiation. qPCR was performed to investigate Sox9, CRTAC1 and COMP gene expressions during differentiation, while confocal microscopy was used to highlight the protein levels of same chondrogenic markers. COMP expression was also assessed using ELISA, whereas scaffold structure during differentiation was analyzed by SEM studies.

CHTβGP was validated as a biocompatible scaffold by MTT, LDH and Live/Dead assays. SEM analysis further confirmed its biodegradability. Chondrogenic markers CRTAC1 and COMP displayed an upregulated profile of gene and protein expression at 14 and 21 days post induction, as compared to the early stages of the experiment, whereas Sox9 highest levels were detected in the first days post induction, proving to be the main inductor of the chondrogenic differentiation process.

Upregulated condrogenic pattern suggests that CHTβGP could be a suitable 3D bioconstruct for cartilage reconstruction applications due to its biocompatibility and capacity to support hADSCs differentiation.

Key words
Cartilage tissue engineering, chondrogenesis, chitosan, β-glycerophosphate, human adipose-derived stem cells (hADSCs), biocompatibility, stem cell differentiation.
Preparation and Evaluation of Biological Properties of Bioimplants Composed of Adipose Derived Stem Cells and Porous Titanium Scaffold (Ti-6Al-7Nb) With HAp/Ca-P surface

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Bone defects in patients with bone cancer present a huge problem especially for patients after resection of the large part of the mandible. Scientists put a lot of effort to find optimal solution for post-resection patients. The aim of our study was to prepare the functional bioimplants composed of human adipose derived stem cells (hASC) and porous titanium scaffolds covered with HAp/Ca-P. Evaluation of biological properties of the scaffolds and their influence on hASC osteogenesis in vitro was performed. Titanium scaffolds were produced as lattice structures from powder of titanium Ti-6Al-7Nb by Selective Laser Melting. Scaffolds’ surface was modified by the immersion process in simulated body fluid. hASC at passage 2, were seeded on the scaffolds and cultured in osteogenic medium supplemented with inorganic phosphate. DNA content measurement (PicoGreen) revealed increasing number of hASC on scaffolds during 6 weeks of culture. Very high cell density led to complete clogging of pores and prevention of free flow of reagents. Therefore cellular metabolic activity measured by XTT assay decreased between 2nd and 6th week of culture. Microscopic investigation indicated proper morphology of cells on the scaffolds and very high cell density after 6 weeks. Cells were present also deep inside the scaffolds. FACS analysis revealed presence of osteonectin (osteogenic marker) after 2 and 6 weeks of culture. Large areas of mineralization were found on cell-seeded scaffolds after 6 weeks of in vitro culture. Summarizing, biological evaluation of bioimplants properties demonstrated cytocompatibility of modified scaffolds and create opportunity for future application in patients with large bone defects.

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The ability to control the behaviour of stem cells, which is regulated by their environment, is an essential goal in the field of regenerative medicine. Since the discovery of dental mesenchymal stem cells (DMSCs) in human teeth their potential for the use in cell-based therapies has been studied intensely. Understanding the migration, proliferation, and differentiation capabilities of various DMSC populations in artificial or natural 3D environments will support the development of new therapeutic approaches for tissue repair. Human dental pulp and dental follicle stem cells (DPSCs/DFSCs) were grown in mono- and co-cultures. Migration and proliferation were analysed by time-lapse imaging and regulatory genes involved in dental cell migration and differentiation were measured by qRT-PCR. DPSCs and DFSCs presented low and irregular migration profiles under mono-culture conditions. In co-cultures, DFSCs showed an increased migration activity and velocity and surrounded the DPSCs. Gene expression profiles of DPSCs and DFSCs were also influenced. Furthermore, DPSCs seeded on 3D silk scaffolds gave rise to mineralized structures, which were increased in the samples grown in spinner flask bioreactors in the presence of osteogenic medium by a factor of 10.5±4.7 when compared to the static control grown in standard culture medium.

The present findings prove that the environment does influence the behaviour of DPSCs and DFSCs, which keep their genetic memory and compete with each other for territory in vitro. Mechanical loading was shown to play an important role in the mineralization process. Future studies will investigate the effect of dentinogenic factors on the behaviour of DMSCs.
Novel Gellan Gum-Bioactive Glass Composite Hydrogels for Tissue Engineering Applications

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Tissue engineering and regenerative medicine is one of the fastest developing fields in science, bridging multiple disciplines together in search of effective innovations. To provide tissue-engineered solutions, as for example for bone regeneration, 3D matrices with appropriate behaviour are needed aiming at mimicking as close as possible the native tissue. A biodegradable polysaccharide gellan gum (GG) matrix reinforced with nano-size bioactive glass (BAG) particles is herein proposed as a promising solution for that purpose acting as a stem cell differentiation template.

GG hydrogels were prepared taking advantage of ionic cross-linking mechanisms to obtain three-dimensional stable structures with a water content of around 4000%. With the aim to further improve its bioactivity, particles of BAG, a bioinorganic material known to be osteoconductive, were incorporated as bioactive filler. Composite hydrogels with four different compositions were prepared by mixing the BAG particles with the GG solution prior jellification. SEM observation of the dehydrated GG hydrogels with BAG particles embedded into the polymer revealed smaller pores, relatively to dehydrated GG hydrogels, that resulted in improved mechanical properties, not only due to the presence of the BAG particles per se, but also due to an additional calcium cross-linking effect provided by BAG. Most importantly, the composite hydrogels depicted a bioactive behavior after immersion in simulated body fluid.

Figure 1: A) GG hydrogel (upper image) and composite hydrogel (lower image); B) microstructure of the dehydrated composite hydrogel; C) detail of the wall of dehydrated composite hydrogel; D) hydroxyapatite formed in simulated body fluid evidences their bioactivity character; E) Modulus of GG hydrogels and GG hydrogels with BAG.

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Demineralized bone matrix (DBM) powder is widely used for bone regeneration. However, difficulties with handling, its tendency to migrate from graft sites, and lack of stability after surgery can limit its clinical use. In this work, the possibility of using a poloxamer carrier to deliver DBM powder in bone defects was assessed in vivo. A putty of 40% DBM (TissueLab, Naples, Italy) and Pluronic was prepared. The effect of DBM-putty on healing was evaluated on transcortical femur lesions (Ø 3 mm) in Wistar rats. Pluronic and unfilled defect were used as reference.

Histological analysis and Micro-CT scans of each femur were carried out to assess the bone healing. Histological analysis of DBM-putty bone defect at 15 day evidenced the presence of numerous osteoblasts and new blood vessels indicating that the DBM-putty supports the physiological healing process. At day 30 and 90 a complete reabsorption of DBM-putty and a bone structure formation comparable with unfilled defects were observed. Absence of host-implant reaction in newly formed bone was assessed at each time-points. The Micro-CT images of bone defect filled with DBM-putty evidenced the formation of bone trabeculae from the medullary region after 15 days with subsequent formation of a dense trabecular network and a thick cortical region at 30 and 90 days respectively.

This work showed that DBM-putty supports natural bone repair in a Wistar rat model allowing new vessels growth and cells infiltration.
Carbon materials are extensively used in hemosorption, enterosorption as well as applique and bandaging material. Biological inertness and high sorption activity can extend possibility their utilization as scaffolds for tissue engineering. In this study we have tested the possibility of carbon material application for scaffold implantation in biological objects. Study of cell behavior in nondegradable scaffold allows to identify the implanted structures in an organism and to track the dynamics of changes in implanted cells. Activated carbon materials with different structures: spherical carbon sorbent, carbon fiber obtained by carbonization of cellulose acetate fibers and multiwalled carbon nanotubes have been used for cell immobilization. Study of cell adhesion activity on carbon materials revealed different degrees of cell immobilization depending on differences in sorption capacity of materials tested, which seems to be associated with specific surface area, pore size distribution and pore volume of the material. The highest potential for cell adhesion (tested on primary EGFP+ cells of transgenic mice, murine immortalized cell lines, human mesenchymal stem cells obtained from umbilical cord and adipose tissue) was observed in carbon fiber (sorption space 0.6-1.5cm³/g, surface area of pores 450-800m²/g). Intraperitoneal implantation of the immobilized transgenic murine cells expressing human insulin into diabetic mice resulted in significant reduction of hyperglycemia.
Decellularized marine sponges are considered to be the promising natural scaffolds for tissue engineering. The aim of this study was to investigate the morphological and functional properties of human adult adipose tissue-derived mesenchymal stromal cells (MSC) within chitin-based scaffolds derived from decellularized marine sponges of Verongida order (*Aplysina fulva* and *Ianthella basta*). MSC were isolated from human adult adipose tissue, expanded in vitro and then seeded into natural scaffolds by the perfusion method. Cell morphology within scaffolds was estimated by staining of MSCs by azure-eosin. Cell metabolic activity was assessed by Alamar blue and MTT tests on 7th and 14th day of culture. Differentiation capacity of MSC within natural scaffolds was observed after the addition of adipogenic and osteogenic inductors. After decellularization and demineralization procedures, sponge skeletons had the macroporous structure formed by intersecting chitin fibrils. After seeding into chitin-based matrices cells attached to the surfaces of the fibrils had flattened fibroblast-like morphology and were able to proliferate. Under the influence of appropriate inductors MSC within 3D scaffolds were able to differentiate into osteogenic and adipogenic lineages. Thus, the application of demineralized and decellularized skeletons of marine sponges *Aplysina fulva* and *Ianthella basta* as scaffolds for MSC provides new opportunities for stem cell based tissue engineering applications.
Hollow-fibre membrane bioreactors (HFMBs) are currently under experimental investigation for use in tissue engineering. HFMBs have great potential to control growth of a clinically-relevant cell population as the surface area for nutrient delivery is large compared to the bioreactor volume. They consist of a cylindrical glass module with an exit port at either end, through which a synthetic hollow fibre is inserted. Cells are seeded in the extracapillary space surrounding the fibre, and culture medium is pumped into the fibre lumen, reaching the cells via the porous fibre walls which protect them from damage due to high fluid shear stresses. We have developed a mathematical model of a simplified, two-dimensional HFMB, with the aim of understanding how to control cell distribution using flow. We solve for the cell distribution, solute concentration and reduced water pressure for a number of experimentally-motivated case studies. These setups consider different solutes and flow regimes, and results demonstrate the range of possible behaviours in each scenario. We observe that since solute gradients induce either spatial heterogeneity in cell proliferation rates (in the case of oxygen), or cell movement due to chemotaxis (in the case of a chemoattractant), a careful balance between advection and these effects is required in order to obtain a uniform cell population. In each case we can therefore predict the dependence of cell distribution and yield on the prescribed flow rate.
The regeneration of traumatised, damaged or lost bone is still a major clinical and socio-economic problem. Bone-tissue engineering approaches involve culturing mesenchymal stem cells (MSCs) on 3D scaffolds designed to mimic the composition, structure, and biomechanics of the native bone matrix. It is now recognised that the ability of MSCs to control other cells through secretion of paracrine/autocrine factors contributes more significantly to tissue repair than their multilineage potential. One of the key challenges in bone-tissue engineering is to understand the host response to implantable engineered constructs. Macrophages play an important role in fracture healing and tissue repair and are mainly involved in the inflammatory response at the early stage upon scaffold implantation. In this context, we proposed to study the ability of the architecture of human MSCs to modulate the secretion of soluble factors involved in fracture healing in a co-culture model with macrophages. To this aim, human MSCs seeded on highly porous polystyrene scaffolds, which provide a 3D spatial environment, and TPA-differentiated THP-1 cells were co-cultured using a transwell insert system. Our data showed that 3D growth of MSCs improved their osteogenic maturation, which was not affected by the presence of THP-1 cells. Compared to isolated macrophages, co-cultures with MSCs resulted in decreased TNF-alpha and RANTES levels increased IL-6, MCP-1, IL-10, PGE2 and VEGF while IL-1 beta and IL-8 were unaffected. Among these factors, 3D architecture of MSCs regulated IL-6, MCP-1, RANTES and PGE2 secretion in our co-culture model. To date, our data suggest that interactions of MSCs in a scaffold's 3D environment are able to modulate the local complex interplay of soluble factors during bone regeneration.
Assessment of Mesenchymal Cells Isolated from Tooth Pulp and Composite Scaffolds Produced by Surface Selective Laser Sintering as Components of Bone Tissue Engineering Constructs.

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We are trying to develop a tissue engineering approach to the reconstruction of bone defects using dental pulp-derived mesenchymal stem cells (MSCs) seeded onto polymer or polymer-mineral scaffolds fabricated from polylactoglycolide with addition of bioactive calcium phosphate ceramics by surface selective laser sintering (SSLS). Cultures of mesenchymal cells were derived from the pulp of human deciduous teeth. Presence of MSCs in the culture was verified by cell surface marker expression profile (CD29+, CD34-, CD44+, CD45-, CD49b+, CD73+, CD90+, и HLA-DR-). Experiments with various materials (brushite-hydroxyapatite cement, hydroxyapatite cement, tricalcium phosphate prepared by the conversion of gypsum, tricalcium phosphate ceramics) were carried out to select those allowing better proliferation and efficient osteogenic differentiation of the cells. Of the materials tested, a greater degree of cell survival and proliferation was observed in case of culturing the cells in the presence of tricalcium phosphate ceramics (Fig. 1, A). Tricalcium phosphate ceramics visibly enhanced osteogenic differentiation as evidenced by the increased number of calcified nodules and increased extracellular matrix production (Fig. 1, B). Based on these results we have chosen to incorporate tricalcium phosphate ceramics into polylactoglycolide scaffolds with SSLS. The scaffolds were then successfully seeded with MSCs (Fig. 1, C). Cells cultured with this material remained viable for a long time, thus providing data confirming the possibility of clinical usage of our technology in the field of bone regeneration.

Fig. 1. A – Changes in the number of viable cells during culture in the presence of different calcium phosphates; B – Accumulation of calcium phosphate in cells during osteogenic differentiation in the presence of tricalcium phosphate ceramic (TCPC) 1 – cells cultured in growth medium; 2 – cells cultured in dexamethasone-supplemented medium; 3 – cells cultured in dexamethasone-supplemented medium in the presence of TCPC; 4 – cells cultured in the presence of TCPC. Alizarin red staining. Magnification ×100; C – composite mineral-polymer scaffold seeded with MSCs. Nuclei stained with DAPI, Magnification ×100.
Poly (γ-glutamic acid)-based Fibrous Scaffolds for Cutaneous Wound Repair

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When skin is severely injured such as in burns and scalds, poor healing and repair often results in scarring and contraction. Although pharmacological interventions and surgical techniques have improved mortality and morbidity, post-burn scars and contractures still present significant challenges and can result in life-long aesthetic, functional and psychological impacts on the patient. Biocompetent biomaterials capable of directing defined cellular activities may present a potential solution to these problems. One such material Poly (γ-glutamic acid) (γ-PGA) has been advocated for the fabrication of materials for biomedical applications. γ-PGA is biodegradable, highly soluble in water due to its polyanionic nature and can be fabricated into derivatives with controlled solubility across a wide pH range and allow for grafting of specific bioactive molecules for the enhancement of its performance1,2. Indeed, γ-PGA has been chemically modified to produce a large variety of functional derivatives and may be engineered as submicron beads or fibres through emulsion and electrospinning techniques respectively1,3. No systematic work has yet been performed on γ-PGA to functionalise it for use in wound healing and contraction reduction applications. The present work reports the optimised functionalisation of derivatised γ-PGA fibres with peptides mimicking basement membrane features (BMM) and platelet derived TGF-β-activation inhibiting peptides (PTAI). The effect that these fibres have on keratinocytes and fibroblast viability, and the differentiation of fibroblasts into myofibroblast is also reported.

All reagents were of analytical grade. Benzylated γ-PGA (γ-PGA-Bn, degree of substitution 77 %) was electrospun into fibrous mats onto glass coverslips (discs) in a random or aligned orientation. The discs were functionalised with BMM and PTAI peptides using carbodiimide chemistry. Briefly The discs were placed in 2 ml of 2-((N-morpholino) ethanesulfonic acid (MES) buffer (0.1 M MES, 0.3 M NaCl, pH 6.5) containing 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC, 4 mM) and N-hydroxysuccinimide (NHS, 10 mM) at room temperature for 30 minutes. The discs were then washed with MES buffer (X3) and then placed into an MES buffer solution containing the peptides (1mg/ml) and allowed to react for at least 4 hours at room temperature under gentle agitation. The reaction was then quenched by the addition of hydroxylamine (10 mM) and washing through MES buffer, distilled water and lyophilisation. Human keratinocytes and fibroblasts were isolated from discarded skin with patient consent and cultured using standard in vitro cell culture methodology. Following treatment with trypsin, a cell suspension of either keratinocyte or fibroblast cells was seeded onto aligned or randomly oriented γ-PGA and PLLA fibres at a density of 2 x 10^5 cells/cm^2. Cells were incubated on fibres for 5 days, fixed, double stained with phalloidin (Alexa fluor 488 phalloidin, Invitrogen, UK) and DAPI and visualized by fluorescence microscopy. Cell viability studies were carried out using ATPLite Assay and MTS assay and cytotoxicity was carried out using LDH assay kit.

Successful derivatisation and functionalisation of the benzylated γ-PGA fibres was confirmed by FTIR. SEM analysis confirmed that fibres were a mean of 260 nm in diameter and were either aligned and random in orientation. Keratinocytes, fibroblasts and actin filaments within these cell types assumed either random or aligned morphology on randomly and aligned oriented fibres respectively (Fig1 A & B). The culture of cells on BMM and PTAI functionalised fibres did not affect cell viability. Analysis of α-smooth muscle
*actin* showed the effect of the peptides on cell attachment, contraction and the differentiation of fibroblasts into myofibroblast. This works demonstrate that partially benzylated γ-PGA can be fabricated into fibrous mats and functionalised with bioactive peptides. The cell and filamentous actin orientation, which is responsible for cell contraction and motility of skin cells can be controlled by fibre orientation. The presence of bioactive peptides allowed for control of specific cellular mechanisms involved in wound healing and contraction. The analysis of α-smooth muscle *actin* expression by fibroblasts provided a measure of fibroblast contractile phenotype in response to fibre constructs with differing properties. As such, these novel fibres are promising potential in controlled cutaneous wound healing applications.

**References**

Adipose-Derived Stem Cells Biocompatibility and Chondrogenic Potential Assessment in Novel Chitosan-βglycerophosphate Scaffolds Designed for Cartilage Reconstruction

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Natural compounds are currently required for the improvement of tissue engineering strategies. This study aims to assess the biocompatibility and the chondrogenic differentiation potential of human adipose-derived stem cells (hADSCs) in contact with a novel chitosan-βglycerophosphate 3D matrix (CHTβGP) designed for cartilage regeneration.

hADSCs in the 4th passage were seeded on CHT surface but cells managed to populate deeper layers of the scaffold, resulting in a 3D culture. This system was evaluated in terms of biocompatibility by quantitative and qualitative assays. Furthermore, the bioconstruct was exposed to chondrogenic induction cocktail for 28 days and the process was monitored after 7, 14 and 21 days of differentiation. qPCR was performed to investigate Sox9, CRTAC1 and COMP gene expressions during differentiation, while confocal microscopy was used to highlight the protein levels of same chondrogenic markers. COMP expression was also assessed using ELISA, whereas scaffold structure during differentiation was analyzed by SEM studies.

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Upregulated condrogenic pattern suggests that CHTβGP could be a suitable 3D bioconstruct for cartilage reconstruction applications due to its biocompatibility and capacity to support hADSCs differentiation.

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Differentiation and Mineralization of Marrow Stromal Cells in Osteon-Inspired Microtubes

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In cortical bone, the aligned collagen fibrils deposited with calcium phosphate nanocrystals are laminated to form the microtubular osteons that resist compression and provide osteoconductivity. Inspired by the nano- and microstructure of osteons, the objective of this work was to synthesize laminated microtubes from aligned poly(lactide-co-glycolide) (PLGA) nanofiber sheets with surface glutamic acid (GLU) sequence for nucleation and growth of calcium phosphate crystals and to test the osteon-inspired microtubes with respect to osteogenic differentiation and mineralization of marrow stromal cells (MSCs). Microtubes with inside diameters ranging from 250 to 800 µm were fabricated. For cell culture studies, sterilized microtubes were conditioned in primary culture media prior to cell seeding. MSCs isolated from rats were seeded in the microtubes at a density of 1x10^5 cells/cm² and cultured in osteogenic medium for up to 28 days. At each time point, the cell-seeded scaffolds were characterized by DNA content, alkaline phosphatase activity (ALPase), calcium content and total collagen content. The average fiber diameter was 100 nm and the average CaP crystal size was <100 nm. The heat-shrunk microtubes had >1 GPa tensile modulus. DNA number, ALPase activity, calcium content and total collagen content of the MSC-seeded microtubes were investigated. ALPase activity, calcium content and total collagen content of the microtubes (MT) was significantly higher than those of the microsheets (MS). The diameter of the microtubes (350 versus 800 µm) did not have a significant effect on osteogenic differentiation of MSCs. The results demonstrate that geometry affects differentiation and mineralization of MSCs.
Auricular Cartilage Repair Using Regeneration of the Abdominal Wall after the Repair of Full-Thickness Defects with a New Composite Prosthesis

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Composite prostheses are designed to achieve optimal tissue integration and at the same time prevent intraperitoneal complications. This study compares the regeneration of the abdominal wall using 3 different composite prostheses.

Defects (7x5cm) created in the abdominal wall of New Zealand rabbits were repaired using a polypropylene Bard™ Mesh (control: n=12), and 3 composites (n=12 each): Physiomesh™ (Phy) (Ethicon), Ventralight™ (Vent) (Bard) and a new composite mesh (Ncm) (Covidien). Six animals per group were sacrificed at 14 and 90 days post-implant. Tissue integration, gene and protein expression of collagens (RT-PCR/immunofluorescence) and macrophages were determined.

14 days after implant all the prostheses became fully infiltrated by loose connective tissue, showing good host tissue ingrowth. Collagen III expression was homogeneously distributed in the neoformed tissue, but staining intensity was greater at 90 days in Phy and Vent, compared to 14 days. Phy showed the faintest collagen I expression, while Vent showed the most intense at 14 days. In the long-term, the pattern was similar but staining and intensity was higher. Collagen 1/3 mRNA showed a similar pattern in Bard and Ncm, with an expression peak at 14 days, significantly reduced by 90 days. Phy showed the opposite pattern with increased expression at 90 days. Expression patterns for Vent remained unchanged. Vent elicited the most intense macrophage response and the other two composites showed similar response at each time point.

Ncm showed accelerated tissue maturation and higher quality of newly formed tissue, rich in mature collagen, along with a low inflammatory response throughout the entire study.
Extraembryonic Tissues – Source of Mesenchymal Stem Cells and Scaffolding Material

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Polish Stem Cell Bank (PBKM), ul. Grzybowska 2/41, 00-131 Warsaw, Poland Tissue engineering (TE) involves the use of cells and materials of natural or synthetic originto restore damaged tissues. Adequate cell type and cell source are vital for successful implementation of TE product in clinic. Placenta and extraembryonic tissues are considered potential source of mesenchymal stem cells and scaffolding material (ECM – ExtraCellularMatrix). Therefore, the aim of this study was to investigate properties of cells and ECM derived from extraembryonic tissues. We simultaneously tested several methods of AMSC (Amniotic Mesenchymal Stem Cells) isolation described in literature and selected ones superior for further analysis. Cells at passage 1 were subjected to detailed characterization based on cell surface antigens. More than 99% of cells were positive for CD29, CD49d, CD73, CD90, CD105, CD166. Above 84% of cells were positive for W5C5 and about 55% for HoxA5. Cells were negative for hematopoietic lineage markers – CD14, CD34, CD45 and endothelial cells – CD31 or for CD36 markers. Percentage of cells expressing pluripotency markers such as Nanog, Oct-4, Sox2, Flizzled-1 was low, not exceeding 4%. Placenta from the same patient was decellularized using sodium dodecyl sulfate solution. Further, ECM was incubated for 24 hours with DMEM at 37°C. Collected supernatant was assessed for the presence of growth factors using ELISA (R&D Systems). We detected presence of TGF-β1 (7613.8 ± 3367.1 pg/g dry weight of ECM), FGF-2 (6357.1 ± 232.8 pg/g dry weight of ECM) and VEGF (35381.8 ± 894.4 pg/g dry weight of ECM). Summarizing, extraembryonic tissues are a rich source of mesenchymal stem cells and scaffolding material offering a microenvironment supportive to the growth and differentiation of cells. POIG.01.01.02-00-022/09
Mammalian central nervous system (CNS) neurons do not regenerate after injury due to the inhibitory environment formed by the glial scar, largely constituted by myelin debris. The use of biomaterials to bridge the lesion area and the creation of an environment favoring axonal regeneration is an appealing approach, currently under investigation. We have been exploring the use of trimethylene carbonate (TMC) and ε-caprolactone (CL) (co)polymers with the final goal of using these materials in the development of conduits to promote spinal cord regeneration. The nanomechanical properties that can be tuned by varying the copolymer composition were found to have a major role in axonal extension.

P(TMC-CL) with a high CL content was found to stimulate neuronal polarization and promote axon elongation, while the CL homopolymer was not able to mediate such behavior. Furthermore, cortical neurons cultured on P(TMC-CL) in the presence of myelin were able to overcome myelin inhibition in comparison with the control condition (glass substrate). This effect was found to be mediated by the glycogen synthase kinase 3β (GSK3β) signaling pathway with impact on the collapsin response mediator protein 4 (CRMP4), suggesting that nanomechanical properties were implicated in this process.

Bi-layer P(TMC-CL) scaffolds were subsequently designed being constituted by a solvent cast film and a layer of preferentially aligned electrospun fibers that are being tested not only to serve as a physical support to promote axonal elongation but also for the delivery of drugs that can modulate the regeneration environment.

Our findings indicate P(TMC-CL) with a high CL content as a promising material for CNS regenerative applications as it promotes axonal growth, overcoming myelin inhibition, while being a versatile material that can be processed into a variety of forms and combined with different therapeutic agents.

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Mouse Compact Bone Derived Mesenchymal Stem Cells Supress Airway Inflammation in Both Chronic and Acute Murine Asthma Model

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Aim: The aim of the present study was to investigate the efficacy of mouse compact bone (mCB) derived MSCs on lung histopathology and lymphocyte proliferation.

Methods: mCB-MCS were isolated from BALB/c mice, characterized and marked by GFP. To generate murine models of chronic and acute asthma, mice were i.p. sensitized with OVA and exposed to aerolized OVA. mCB-MSCs (2.5x10⁵ cells) were administrated i.v. after last nebulization. Mice were sacrified, and splenocytes and lung lymphocytes were isolated and marked with CFSE. Cells stimulated with OVA (40µg/ml) were cultured under suitable conditions for 5 days. Flow cytometric analysis and histopathological examination of lungs were evaluated. In histopathological analysis, the measurements were performed from minimum 5 points of each airway and mean values were calculated. Goblet cells stained with PAS enumerated in 2500 cells.

Results: In sections stained with H&E, the distal [without MCS chronic:29.9µm acute:32.03µm; with MSC chronic:13.3µm acute:12.25µm] and proximal [without MCS chronic:42.6µm acute:28.97µm; with MSC chronic:17.4µm acute:18.9µm] airway epithelial thicknesses were observed to decrease in both mouse models. Likewise, in sections stained with PAS, a significant reduction in number of hyperplasic goblet cells in the proximal [without MSC chronic:140 acute:1200; with MSC chronic:4 acute:211] and distal [without MSC chronic:55 acute:118; with MSC chronic:0 acute:0] airways was observed. Moreover, in the CFSE staining experiment, CB-MSCs inhibited lymphocyte proliferation in both asthma model.

Conclusion: The results reported here provide that mCB-MSCs may provide powerful alternative therapeutic for the treatment of chronic and acute asthma.
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