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Oral Sessions

BINDING OF BMP-2 TO TITANIUM IMPLANT MATERIALS

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Introduction

Bone morphogenetic proteins (BMPs) are important factors in bone formation and repair. However, the proteins are highly soluble *in vivo* and are quickly removed from their site of application [1]. Therefore, self-assembled monolayers (SAMs) of silanes have been employed to couple bone morphogenetic proteins (BMPs) to the titanium surfaces [2]. Recently, phosphonic acid molecules were used for SAM formation on titanium. Advantages of these monolayers in comparison to silanes SAMs are the higher hydrolytic stability [3] and that no surface conditioning (i.e. acid treatment) is required in order to obtain high coverage. Gawalt et al. developed a simple but effective route to immobilize stable phosphonic acid monolayers onto titanium surfaces [4]. In this study, we investigated the possibility to bind BMP-2 to activated monolayers of hydroxyundecyl phosphonic acid and carboxyundecyl phosphonic acid on titanium. X-ray photoelectron spectroscopy (XPS) and time-of-flight secondary ion mass spectroscopy (ToF-SIMS) were used to characterize the chemistry and structure of the different surface modification steps.

Materials

Ti90/Al6/V4-Pieces (10x10mm) (Goodfellow) were ground with 800, 1200 and 2500 grit SiC-paper, polished with colloidal silica (type SBT) and a Multitex SBT polishing cloth, then sonicated with appropriate solvents. The hydroxyundecyl phosphonic acid and carboxyundecyl phosphonic acid monolayers were prepared according to literature [4]. The surfaces were activated using CDI and NHS, respectively. The activity of the surfaces was tested using Trifluoroethylamine (TFEA) hydrochloride. BMP-2 was coupled to the activated surfaces from solution in MES-buffer. Bound BMP-2 was detected by ELISA. XPS was obtained using a Kratos AXIS Ultra DLD instrument. ToF-SIMS spectra were acquired on a Physical Electronics PHI 7200 time-of flight spectrometer.

Results and Discussion

The formation of monolayers of hydroxyundecyl phosphonic acid and carboxyundecyl phosphonic acid was confirmed by XPS and SIMS measurements. In XPS carbon, oxygen and phosphorus were detected close to the theoretical value. Angle dependent composition scans revealed an orientation of the phosphorus towards the surface. Upon activation, the nitrogen content of both surfaces increased, indicating a successful reaction. Furthermore high resolution carbon scans revealed the

characteristic peakshifts of the amide carbonyl of the NHS and CDI respectively. The binding of TFEA to these activated surfaces was confirmed by the presence of fluorine in the composition scan and the appearance of a high energy (292 eV) carbon peak due to the CF₃ group. (see Figure 1) SIMS measurements confirmed the monolayer formation as well. BMP-2 was coupled successfully to the surfaces as evidenced by ELISA. First *in-vivo* experiments are under way.

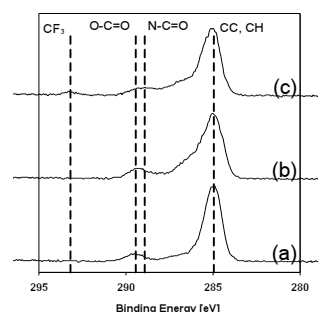


Figure 1. HighRes C1s spectra of COOH terminated monolayer on titanium (a) after activation with NHS (b) and the binding of trifluoroethylamine (c)

Conclusion

Overall this approach is a promising way to bind proteins like BMP-2 to titanium to improve its bioactivity.

Acknowledgements

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QUANTITATIVE KINETIC ANALYSIS OF GENE EXPRESSION DURING HUMAN OSTEOBLASTIC ADHESION ON ORTHOPAEDIC MATERIALS

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Introduction

Little information was found in the literature about the expression of genes specific of cellular adhesion molecules on calcium phosphate materials although more were found on titanium-based substrates. Hence, the goal of this work was to study by a kinetic approach from 30 minutes to 4 days the adhesion of human osteosarcoma Saos2 cells on microporous (mHA) and non microporous hydroxyapatite (pHA) in comparison to polished titanium. Our strategy associated the visualization of adhesion proteins inside the cells by immunohistochemistry and the quantitative expression of genes at mRNA level by real-time PCR. The cell morphology was assessed using scanning electron microscopy and the number of cells thanks to biochemical techniques.

Methods

Microporous hydroxyapatite (mHA) discs were provided by BIOCETIS s.a. (France). They were obtained by a humid method using commercial powders of HA (Transtech, USA). The discs were then sintered at high temperature (1250°C). mHA displayed 12.5% of interconnected microporosity. Non microporous hydroxyapatite discs (pHA) were prepared using a laboratory-prepared HA powder which was pressed by uniaxial pressure of 80 Mpa. Discs were sintered at 1220°C and polished with SiC paper. Mirror-polished pure titanium discs, were obtained from the Laboratoire Matériaux (ENSAM, Lille, France). SaOs2 cells were cultured on samples for 30 min, 1h, 4h, 24h or 4 days. Cells on samples were either treated for immunofluorescence, for adhesion assay or for protein and RNA extraction. For these last measurements, cells on each sample were immersed in 0.25 ml of Extract-All (Eurobio, France) and proceeded for protein and RNA extraction. The expression of genes involved in bone cell adhesion and differentiation was quantified by real-time PCR.

Results

The cellular attachment was the highest on mHA from 30 minutes to 24 hours although the cell growth on mHA was the lowest after 4 days. Generally, the SaOs-2 osteoblastic cells morphology on mHA was radically different than on other surfaces with the particularity of the cytoplasmic edge which appeared un-distinguishable from the surface. The revelation by specific antibodies of proteins of the cytoskeleton (actin) and the focal adhesions (FAK, phosphotyrosine) confirmed that adhesion and spreading were different on the 3 materials.

The actin stress fibers were less numerous and shorter on mHA ceramics. Cells had more focal contacts after 4 hours on mHA compared to other substrates but less after 24 hours. The highest values of total proteins were extracted from mHA at 0.5 and 24 hours and from pHA at 1, 4, and 96 hours. The αv and $\beta 1$ integrin, actin, FAK, and ERK gene expression were found to be different with adhesion time and with materials. C-jun expression was comparable on mHA, titanium and plastic but was largely higher than on pHA at 0.5 and 1 hour. On the contrary, c-fos expression was the highest on pHA after 0.5 hours and the lowest after 1 hour. This difference between c-fos and c-jun expression on pHA after 0.5h could be related to the fact that these two genes may differ in their signalling pathways. The expression of the alkaline phosphatase gene after 4 days was lower on mHA compared to other materials demonstrating that the microstructure of the mHA ceramic was not favourable to SaOs-2 cells differentiation (Figure 1).

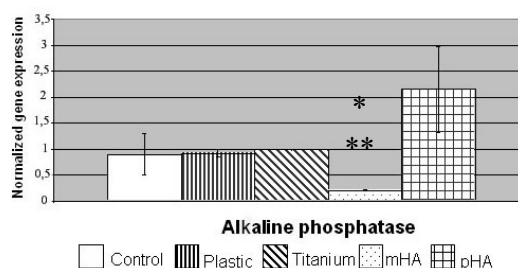


Figure 1: Normalized gene expression of alkaline phosphatase on plastic, titanium, mHA, pHA at 96h. Plastic/mHA (*); $p=0.02642$ and for titanium/mHA (**); $p=0.00886$.

Discussion and Conclusions

Finally, it was demonstrated in this study that HA and titanium surfaces influence as well gene expression at early times of adhesion as the synthesis of adhesion proteins but also proliferation and differentiation phases. The signal transduction pathways involved in adhesion of SaOs-2 cells on HA and titanium were confirmed by the sequential expression of αv and $\beta 1$ integrins, FAK, and ERK genes followed by the expression of c-jun and c-fos genes for proliferation and alkaline phosphatase gene for differentiation. These results are a new demonstration that adhesion of cells on materials is linked to their nature, their protein adsorption capacity, their microstructure, and that it influences further cellular proliferation and differentiation phases.

CELL-MATRIX INTERACTIONS AND SKELETAL DEVELOPMENT

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Introduction

The mammalian skeleton forms via two distinct ways: mesenchymal cells either directly differentiate into bone producing osteoblast (intramembranous ossification) or first lay down cartilaginous precursors, which are subsequently replaced by bone (endochondral ossification). Skeletal morphogenesis is characterized by the expression of a special set of extracellular matrix (ECM) proteins. Cartilage ECM is enriched in type II collagen and aggrecan, which give tensile strength and resistance against compression to the tissue. In addition, the ECM provides instructive environmental clues for proper bone development by distributing growth factors and interacting with cells. Interactions between chondrocytes and the extracellular matrix are important for cartilage homeostasis regulating various cellular functions such as anchorage, survival, proliferation, matrix biosynthesis and degradation. Chondrocyte adhesion to cartilage ECM molecules is primarily mediated via the integrin family of $\alpha\beta$ -heterodimeric transmembrane receptors. Chondrocytes mainly express $\beta 1$ and αv integrins, which mediate adhesion to collagen II and fibronectin. Upon adhesion, ligated integrins are recruited to focal adhesions (FAs) together with adaptor proteins and kinases, such as integrin-linked kinase (Ilk), and initiate downstream signalling events. Cdc42, a member of the family of Rho GTPases, is another intracellular molecule involved in signalling from integrin receptors regulating actin dynamics and cell cycle progression.

Materials

Gene targeting experiments in mice are excellent tools to study the molecular mechanism of skeletogenesis. In the recent years, we have generated a series of conventional and conditional knockout mice lacking specific ECM proteins (e.g. fibronectin, FN), adhesive receptors ($\beta 1$ integrins and $\alpha 10\beta 1$ integrin) and intracellular signalling molecules (Ilk, Cdc42) in cartilage, and their skeletal phenotypes were analysed.

Results and Discussion

Chondrocyte-specific inactivation of the $\beta 1$ integrin and *Ilk* genes (Aszodi et al., 2003; Grashoff et al., 2003) results in perinatal chondrodysplasia with severely disorganized growth plates. The phenotypes of these mice were accompanied by various chondrocyte abnormalities such as shape change, adhesion, actin and proliferation defects. In addition, $\beta 1$ integrin- but not Ilk-deficiency leads to impaired cytokinesis and assembly of collagen II network implicating that Ilk only partially mediates $\beta 1$ function in chondrocytes. Interestingly, the lack of fibronectin in cartilage has no apparent impact on endochondral bone formation (Aszodi et al., 2003) suggesting that chondrocyte adhesion to FN mediated by $\alpha 5\beta 1$ and $\alpha 3\beta 3$ integrins is dispensable for cartilage

function. On the other hand, deletion of the collagen-binding $\alpha 10\beta 1$ integrin leads to a mild growth plate dysfunction (Bengtsson et al., 2005) indicating that this integrin receptor is important but not essential for skeletogenesis and its loss might be compensated by other collagen-binding $\beta 1$ integrins.

To explore the function of $\beta 1$ integrins in adult articular cartilage (AC), we have deleted the $\beta 1$ integrin gene in early limb bud mesenchymal cells. Mutant mice survive and show AC abnormalities of the long bones of the appendicular skeleton. The defects are characterized by a thickening of the AC, chondrocyte clustering, reduced cellularity, altered deposition of collagen X and fibronectin, and the lack of clear tidemarks. At the ultrastructural level, chondrocyte shape change, frequent binucleation, enlarged pericellular matrix compartments, and large collagen bundles near the chondrocyte surface were evident in mutant AC. Compared to wild type AC, surface irregularities and softening of mutant AC were found using atomic force microscopy. The structural abnormalities were accompanied by reduced activation of MAP kinase pathways and reduced MMP13 expression. Finally, we addressed the role of Cdc42 during skeletogenesis by deleting the *Cdc42* gene in limb bud and cranial mesenchymes. In the appendicular skeleton of mutants, the cartilaginous condensations of the long bones were shortened and broadened, the subsequent hypertrophic chondrocyte differentiation, mineralization and the formation of the primary as well as the secondary ossification centres were delayed in mutants. These abnormalities were accompanied by delayed recruitment of MMP-9 positive pre-osteoclasts at the vascular invasion zone and a progressive proliferation defect of growth plate chondrocytes. Cdc42-deficient chondrocytes show abnormal spreading and cytoskeletal organization *in vitro*. In the calvaria, the intramembranous ossification is greatly retarded resulting in wide sutures and open fontanelles in mutants.

Conclusions

All together, our results demonstrate the critical requirement of integrin-mediated cell-matrix interactions in skeletogenesis and identify several molecular mechanisms mediating $\beta 1$ integrin functions in chondrocytes.

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DENDRITIC CELLS AT THE BIOMATERIAL/HOST INTERFACE

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Since biomaterials are used as vehicles for biological components in combination products, it is important to clarify the role of the biomaterial in potentiating any immune response towards the biological component due to a biomaterial adjuvant effect. In tissue engineering applications, immune responses are to be minimized while vaccine strategies seek to enhance the protective immune response. We have shown that poly(lactic-co-glycolic acid) (PLGA) acts as an adjuvant in the immune response against co-delivered antigen¹, depending on the form of the PLGA². As adjuvants act through the maturation of dendritic cells (DCs)^{3,4}, we have examined the differential effects of various biomaterials on DC maturation to assess their adjuvant effect. Furthermore, we are examining the pattern recognition receptors (PRRs) which may be involved in mediating DC recognition and response to biomaterials, particularly focusing in toll like receptor-4 (TLR4). We are also characterizing the biomaterial-associated molecular patterns which may be recognized by the PRRs. DCs may recognize and respond to biomaterials either indirectly through the adsorbed protein layer, for example through carbohydrate modifications of these proteins or through carbohydrates inherent in the biomaterial chemistry using PRRs to initiate an innate immune response.

Materials

Immature DCs (iDCs) were derived from human peripheral blood monocytes by culturing with GM-CSF and IL-4. The effect of biomaterial contact on maturation was studied by culturing iDCs with biomaterials in the form of microparticles (MPs) or films. The following biomaterials were tested: agarose; 75:25 PLGA; chitosan, alginate, hyaluronic acid. The extent of DC maturation was assessed by evaluating the resultant morphology, expression of co-stimulatory and MHC molecules, release of inflammatory cytokines, and allostimulatory function in a mixed lymphocyte reaction (MLR), as compared to iDCs (negative control) and lipopolysaccharide (LPS)-matured DCs (mDCs) (positive control). The requirement of direct biomaterial contact for DC maturation was examined using a transwell assay. The contribution of MP phagocytosis to DC maturation was evaluated using PLGA or agarose MPs of phagocytosable (2µm) and non-phagocytosable (20µm or 30µm) sizes with constant, but varied exposed surface area. Maturation of C57BL/6 bone marrow (BM)-derived DCs upon biomaterial contact was confirmed. The role of TLR4 in biomaterial-induced maturation of DCs is being evaluated by using mice deficient in TLR4 function as sources of BM-derived DCs or HEK293 cells stably transfected to express TLR4 (InvivoGen, San Diego, CA) for treatment with biomaterials and maturation effects compared to controls. Enzyme linked lectin assays using

plant lectin probes were used to elucidate carbohydrate modifications, recognized by the mannose receptor, of adsorbed proteins on alkanethiol self-assembled monolayers (SAMs) of defined chemistries.

Results and Discussion

There was a differential effect of the biomaterial on which iDCs were cultured on the extent of DC maturation; chitosan or poly(lactic-co-glycolic) acid (PLGA) but not agarose, alginate or hyaluronic acid films⁵ supported DC maturation. Culturing iDCs with PLGA MPs or film (but not agarose) resulted in cell morphology similar to mDCs, and expression levels of co-stimulatory and MHC molecules between those exhibited by iDCs and mDCs⁶. The increase in DC marker expression induced by PLGA MPs required direct contact of DCs with the MPs⁶. DCs cultured with PLGA or agarose MPs or films secreted several proinflammatory cytokines in a MP-dose dependent manner; higher levels from DCs treated with PLGA than agarose MPs. MLR results showed that iDCs cultured on PLGA films were more efficient at inducing allogeneic T cell proliferation than iDCs cultured on agarose films. DCs cultured with PLGA or agarose MPs of the different sizes did not change their expression level of mDC markers, implying that phagocytosis was not the main contributor to the MP-induced DC maturation but exposed biomaterial surface area was. Different SAM surfaces were associated with differential carbohydrate profiles. NH₂ SAM surfaces had highest amounts of carbohydrates and CH₃ SAM surfaces the lowest. DCs cultured on NH₂ and COOH SAM surfaces were more mature than those cultured on CH₃ SAM surfaces. Murine DCs from C57BL/6 mice matured upon contact with PLGA MPs or films.

Conclusions

These studies provide insight into design criteria as well as immunomodulatory strategies for biomaterials for a range of applications.

Acknowledgements

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ASSEMBLY AND MECHANOSENSORY FUNCTION OF FOCAL ADHESIONS: EXPERIMENTS AND MODELS

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Focal adhesions as mechanosensors: a physical mechanism

Cells adhere to the extracellular matrix (ECM) via transmembrane integrin family receptors, which together with numerous associated "plaque proteins" form focal adhesions (FAs) linking the ECM to the actin cytoskeleton. FAs are both adhesion and signal transduction organelles, informing cells about the state of the ECM. We have shown that FAs function as touch receptors responding to the mechanical characteristics of the microenvironment [1].

In our theoretical studies, we demonstrated that the major features of the FA mechanosensitive behavior can be explained by thermodynamic principles, which govern self-assembly of molecules into an aggregate subjected to pulling force. Elastic stresses generated within a protein complex decrease the chemical potential of the aggregated molecules relative to the pool of non-assembled molecules. This means that self-assembly of proteins is favored when pulling forces act on the aggregate and disfavored when these forces are relaxed. Considering various types of linkage between the aggregate and the substrate, we predicted different modes of FA assembly and disassembly and showed that the suggested model accounts for the major types of FA behavior observed experimentally [2]. Thus, this model based on very general assumption allows to explain qualitatively the FA mechanosensitive behavior. A hierarchy of diverse signaling circuits shown to be involved in the focal adhesion mechanosensitivity could be build up as a superstructure onto this basic mechanism.

A mechanism of force-driven focal adhesion assembly can be based on the formin function

Signal from small G-protein RhoA is required for the formation of mature FAs and the associated actin filament bundles (stress fibers). Our studies showed that formin homology protein Dial1 is a downstream target of Rho that mediates force-induced FAs formation (reviewed in [1]). More recent experiments with Dial1 knockdown by siRNA (L. Carramusa et al.) confirmed that Dial1 is necessary for the transformation of initial focal complexes into FA and/or for further elongation of FA.

Analysis of formin interaction with actin indicates to a possible direct involvement of formins in the functioning of cellular mechanosensory units. Specifically, theoretical consideration predicted a novel phenomenon, the force-driven polymerization of actin mediated by proteins of the formin family [3]. Formins localize to the barbed ends of actin filaments, but, in contrast to the regular capping proteins, allow for actin polymerization in the barbed direction (the so-called "leaky" or "processive" capping

mechanism). We proposed that the mechanism of leaky capping is based on the elasticity of the formin dimer [3] or, more precisely, elasticity of the formin dimer/barbed end complex [3, 4]. The phenomenon of force-driven actin polymerization is a direct consequence of the phenomenon of leaky capping of actin filaments by formins. We showed that if a pulling force is applied to the formin capping the filament end, the elastic mechanism drives filament growth. Specifically, a moderate pulling force of ~3.5 pN (which can be developed by a single myosin molecule) reduces the critical concentration by an order of magnitude [3].

Cross-talk between focal adhesions and microtubules. A novel role of Dial1 formin

We have shown that Dial1 activation promotes microtubule interactions with FAs [5]. Thus, Dial1 plays a dual role in the regulation of FAs. First, it is necessary for the FA growth that is induced by force. Second, Dial1 promotes microtubule targeting to FAs, which locally inhibit myosin II-driven contractility and facilitates FA turnover [5]. Dial1 dramatically suppresses microtubule plus end growth, in an actin dependent manner [6]. These and other results [6] suggest that Dial1 coordinates the activities of two major cytoskeletal systems, actin and microtubules, in the process of formation and turnover of FAs.

Acknowledgements

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Skeletal stem cells

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Skeletal stem cells (SSCs; popularly referred to as “mesenchymal” stem cells) are found in the clonogenic subset of adherent bone marrow stromal cells. They can be prospectively isolated using a defined set of antibodies recognizing surface epitopes, and their multipotency and self-renewal can be directly demonstrated using appropriate *in vivo* transplantation assays. As proven by direct comparison of phenotype *in vivo* and *ex vivo*, adventitial reticular cells at the abluminal side of sinusoids can now be identified as the *in vivo* counterpart of clonogenic cells. Cell strains generated from SSCs *in vitro* form bone, fat, fibroblasts of donor origin when transplanted ectopically in the immunocompromised mouse, or orthotopically as in preclinical models of bone tissue engineering. In addition, they self-renew into adventitial reticular cells in the heterotopic chimeric bone marrow, from which they can be secondarily isolated in culture (self-renewal).

To prove chondrogenic potential, alternate assays are required, either *in vitro* or *in vivo*. Adventitial reticular cells in the bone marrow are a local variation of cells called pericytes in other tissues, which can in turn be isolated and probed *in vitro* and *in vivo* using approaches conceptually similar to those employed for bone marrow stromal cells. Tissue specific pericytes share the same phenotype, are clonogenic, but exhibit a tissue specific differentiation potential. A general picture emerges outlining the nature of “mesenchymal stem cells”, their origin, mechanisms of their establishment in post-natal tissues, and their function, providing for the first time a rational guideline for their prospective clinical use.

COLLAGEN MATRIX COMPOSITION AND STRUCTURE - INFLUENCE ON BINDING, RELEASE AND ACTIVITY OF TGF- β , BMP-2 AND BMP-4

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Introduction

Components of the extracellular matrix (ECM) can be used as carriers for growth factors. They also provide a microenvironment that can directly influence cells and thus be benefactory to integration and healing. In this context mainly collagen type I has been used, as the protein is the main component of the ECM. Varying the both structure and composition of such collageneous matrices can offer a way of affecting both cell response and growth factor binding.

The collagen matrix can be changed in a number of ways. Among these methods is the inclusion of non-collagenous components with specific functions (i.e. decorin as collagen and TGF- β binding), the cross-linking of matrices to influence degradation and the release of integrated factors, the stacking of differently composed layers to generate graded structures, the presentation of matrix components/ growth factors at different time points.

Using such different approaches, it may be possible to influence the interaction with growth factors and adherent cells, allowing an adaptation of the artificial ECM coating of implants to specific requirements of the host tissue.

Materials and Methods

Experiments were performed on sand-blasted Ti6Al4V samples with a diameter of 10 or 16 mm. Employed proteins where commercially available collagen I bovine (IBFB) or porcine (Colbar), decorin, and chondroitin sulfate (Sigma) as well as the growth factors TGF- β 1, BMP-4 and BMP-2 (R&D). Modified hyaluronic acids were provided by Innovent, Jena. In case of cross-linking EDC was used. Desorption experiments were conducted in cell culture medium with 2 % serum at 37°C. Growth factors were quantified by Elisa.

Cell culture studies were performed with primary osteoblastic cells from rat calvariae. Determined parameters were ALP-activity and collagen synthesis.

For the BMP-Bioassay C2C12 cells were seeded on substrata differing in composition of the collagen matrix adsorbed to titanium alloy and ALP induction was measured.

Results and Discussion

The interaction of growth factors with the collagen matrix depends on several factors. One of them is the matrix composition. Including decorin and chondroitin sulfate into the matrix increases both binding and desorption of TGF- β and BMP-4, respectively, between 10 % - 20 %, indicating an interaction between these

components as is already described for TGF- β . A more significant influence on the release can be achieved by using a layered matrix architecture, or by integrating the growth factors during the fibrillogenesis into the collagen matrix. Especially for BMP-4 the detectable amount in the latter case increased by a factor of 2 to nearly 100 % of the initially bound amount. For TGF- β the differences were less obvious, here in all cases of layered matrices an increase in the signal by 2 – 5 % could be detected.

Hyaluronic acids modified with anionic groups influenced growth factor binding to a certain degree.

Layering the matrix delayed growth factor desorption though the burst release character was retained. In the combined application of BMP and TGF, this delay was already found to increase the ALP induction in C2C12 cells and primary rat osteoblasts.

The results for BMP-2 compared to BMP-4 were an increased adsorption (detected by Elisa), both on collagen and collagen-CS matrices, but a reduced activity of the adsorbed growth factor as measured in the bioassay. The origin of the collagen type I – (bovine/porcine) did not seem to affect these processes significantly. In the case of BMP-4, over 80% of the desorption took place in the first 24 h, while the detectable amounts of BMP-2 after 300 h desorption were less than 10 % of the bound amount. After 300 h no activity remained detectable for either BMP.

Conclusions

Growth factor binding to and release from a matrix composed of components of the extracellular matrix adsorbed to titanium surfaces can be influenced by inclusion of non-collageneous components specific to the growth factor. The architecture (layering) of the matrix can affect release kinetics as well as the measurable activity, something that may be attributed to a protective effect of the matrix. Using two growth factors, these differences can be used to enhance the effect of the factors. Crosslinking stabilizes the matrix, but not necessarily with positive effects for the adsorbed growth factors as their activity was found to be reduced. The matrix characteristics thus play an important role in the activity of growth factors, though the behavior of different growth factor preparation can vary significantly in this respect.

Acknowledgements

This work was supported by the Deutsche Forschungsgemeinschaft (DFG); porcine collagen was provided by Biomet Europe.

HIGH RESOLUTION 3D FIB-SEM MICROSCOPY OF CELL MONOLAYERS ON BIOCOMPATIBLE MATERIALS FOR BIOELECTRONIC DEVICES

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Introduction

A close contact between living cells or tissue and surfaces of bioelectronic devices like micro electrode arrays (MEA) or neuroprostheses is a crucial parameter for the signal transduction from electrodes to cells and vice versa. Great efforts have been made in the past to investigate ultrastructural details of model systems of this bio-technical interface. Ultramicrotomy of Araldite replicas of thin micro structured metal surfaces was used to investigate cell adhesion by transmission electron microscopy [1]. But this method gives only reliable results for very thin metal layers on materials like Araldite which are compatible to ultramicrotomy. To get access to the interface between hard biomaterials and soft biological systems we have therefore developed a new preparation method.

Sample Preparation 1: Embedding

To obtain high-resolution images from details of the technical substrate, the biological material and the interface in between we combined conventional fixation and embedding techniques, Focused Ion Beam (FIB) technology and low voltage Field Emission Scanning Electron Microscopy (FESEM).

3D FIB-SEM Microscopy

FIB preparation and SEM imaging was performed in a crossbeam 1540 XB microscope (Carl Zeiss, Oberkochen). This FIB-SEM microscope allows an easy selection of the region of interest, fast milling at selected areas and SEM imaging of the FIB prepared micro block face [2]. By serial sectioning at this micro block face (Figure 1), layers with a thickness down to 20 nm are removed by FIB milling while the new block face is imaged in real time with the SEM (live milling).

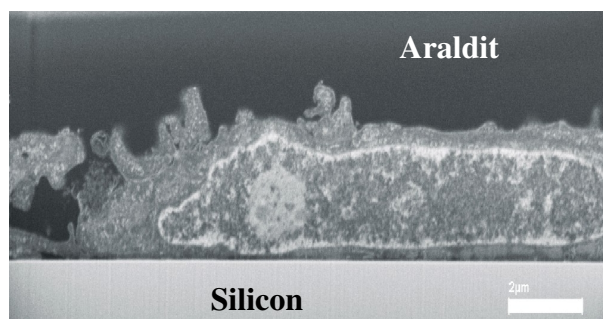


Figure 1: SEM image of the interface between a single cell and a silicon substrate coated with a thin functional layer of titanium.

Sample Preparation 2: Cryo Techniques

Besides embedding, cryofixation of cell monolayers on thin substrates with plunge freezing in propanol was established. Using a cryo transfer system (Baltec VCT 100) and a cryo stage, the frozen hydrated samples may be further processed by milling and imaging inside the cryo-FIB-SEM microscope (Figure 2).

Plunge freezing offers several advantages compared to the embedding technique. Cryo-fixation is a fast process, so the chemical composition of the cell monolayer is frozen by this rapid fixation process.

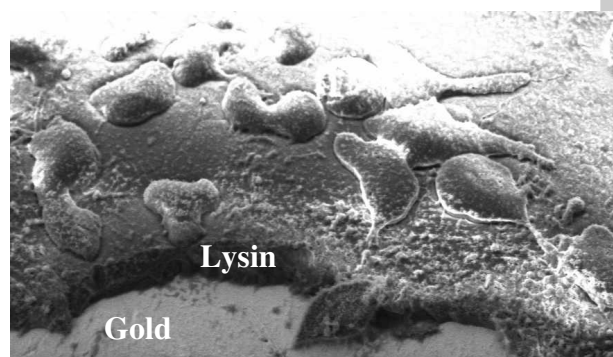


Figure 2: Cryo-SEM of native human vascular endothelial cells grown on a lysine coated gold substrate. A part of the lysine layer was removed by in-situ micro fracture with a small needle.

Conclusion and Outlook

3D FIB-SEM microscopy of the interface between cell monolayers and different biomaterials offers deeper insight to the process of cell growth and adhesion on this materials. The influence of functional layers like thin coatings on this materials may now be systematically investigated. Cryo techniques allow a fast preparation of the cell monolayers and opens the potential for a chemical analysis by secondary ion mass spectroscopy which is currently under development in our lab.

Acknowledgements

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MECHANOREGULATION AT THE CELLULAR AND MULTICELLULAR SCALES

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Introduction

Cellular behavior within multicellular organisms is rigorously controlled by many dynamic cues in the surrounding cellular microenvironment. In particular, adhesive interactions with the ECM and with neighboring cells together coordinate growth, migration, and differentiation. Using microfabrication approaches to engineer cellular microenvironments, we are examining how adhesive cues cooperate to control basic processes such as proliferation and differentiation. Our findings suggest that cells integrate biochemical and mechanical cues from their microenvironment to modulate intracellular signaling pathways and cytoskeletal tension, leading to changes in cellular function. These studies may have a significant impact on our understanding of tissue development and the targeting of specific pathways in disease.

Materials and Methods

Microcontact printing (μ CP) is a technique that enables the modification of biomaterials surfaces to control cell interactions with the underlying ECM and neighboring cells (Fig. 1). A PDMS stamp is coated with ECM

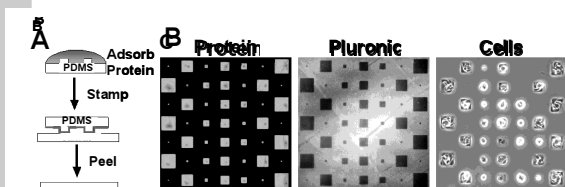


Fig. 1. Microcontact Printing

protein, which is dried and stamped onto a substrate such as glass, polystyrene, or PDMS. A non-adhesive detergent (e.g. Pluronic) is then adsorbed onto the unprinted regions of the surface. Cells adhere to the ECM-stamped regions, but not the detergent-blocked regions. By creating patterns with specific geometries, both cell-ECM and cell-cell adhesion can be controlled.

Results and Discussion

Using micropatterning techniques, we have demonstrated that cell spreading directly regulates cell proliferation and apoptosis [1] (Fig. 2A, B). Recently, we have also demonstrated that spreading affects the commitment of human MSCs to a bone or fat lineage [2] (Fig. 2C). These effects appear to be dependent cytoskeletal tension and Rho GTPase signaling.

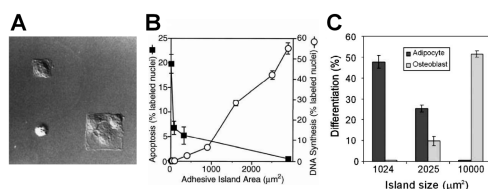


Fig. 2. Cell spreading regulates proliferation and differentiation

We have also used patterning tools to create bowtie-shaped agarose microwells, which allow the control of both spreading and cell-cell adhesion (Fig. 3). One cell adheres to each half of the bowtie and forms a cell-cell contact with a neighboring cell through the center of the bowtie. Using this system, we have observed that cell-

cell contact stimulates proliferation when cell-ECM adhesion remains unchanged. This increase in proliferation has been observed for endothelial and epithelial cells, and depends on cadherins, the major protein within adherens junctions [3].

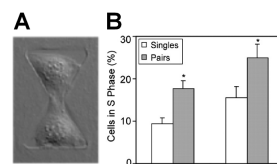


Fig. 3. Cell-cell contact regulates proliferation

Using PDMS as a deformable substrate, we have generated a device consisting of an array of microneedles that detects cellular forces. Cells adhere to and pull on the tips of these needles, which are coated with ECM by μ CP (Fig. 4). The deflection of the needles is determined by forces exerted by the cell on the underlying substrate. Using this device, we have demonstrated that both cell spreading and multicellular organization regulate cellular forces [4, 5].

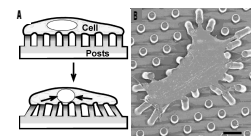


Fig. 4. Micropost array-detectors

Conclusions

We have demonstrated using microfabrication tools that adhesive cues within the cellular microenvironment are important regulators of intracellular signalling pathways and cytoskeletal tension, which together modulate cell proliferation and differentiation. Development of new technologies and further biological studies are needed for a complete understanding of how cells integrate the numerous dynamic cues in their three-dimensional microenvironment.

Acknowledgements

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HIGH PERFORMANCE OF MICRO MACROPOROUS BIPHASIC CALCIUM PHOSPHATE MATRICES FOR BONE TISSUE RECONSTRUCTION AND BONE TISSUE ENGINEERING

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Introduction

The development of CaP ceramics for bone graft involved a better control of the process of biomaterials resorption and bone substitution particularly to optimize calcium phosphate matrices for tissue engineering and bioactive factor carrier. Bone graft materials biomaterials are largely represented by calcium hydroxyapatite, HA; tricalcium phosphate, TCP; and macroporous biphasic CaP, MBCP. The concept based on biphasic CaP ceramics is achieved by an optimum balance of the more stable phase of HA and more soluble TCP. The material is soluble and gradually dissolves in the body, seeding new bone formation as it releases Ca and P ions into the biological medium. These bioceramics are largely used for bone reconstruction and will be specially optimized for combination with bone marrow during surgery or for bone tissue engineering using STEM cells. We have optimized matrices in terms of their physico-chemical and crystal properties; to improve cell colonization and to increase kinetic bone ingrowth. The fast cell colonization and resorption of the material are associated to the interconnected macropores structure which enhanced the resorption bone substitution process. The micropore content involved biological fluid diffusion and suitable absorption surfaces for circulating growth factors.

Materials and Methods

Interconnected MicroMacroporous Biphasic CaP (MBCP2000™, CE123, Biomatlante manufacturer) was an improvement of the technology of macropores developed long time ago¹ to replace classical naphthalene use. Shortly, CaP deficient apatite CDA, were associated to a mixture of selected particles of naphthalene and sugar. After isostatic compaction, the block was sintered according a specific process of sublimation/calcination. The obtained bioceramics was characterized using X-Rays, FTIR, X-rays microtomography, permeability, Hg microporosimetry, BET specific surface area, mechanical test, and SEM. Cylindrical samples of 6mm in diameter and 8 mm length were implanted in femoral epiphysis of New Zealand rabbits and compared to classical MBCP™ as reference (12 rabbits, 24 implantation sites). After 6 and 12weeks, implants were processed for histology and SEM using image analysis.

Results and Discussion

The density was 0.75 for MBCP2000 and 0.83 for MBCP. The crystal size is 0.5 to 1.5 µm and the specific surface area was 1.6 and 1.7m²/g for MBCP2000 and MBCP respectively. Compression test showed 4MPa and 6MPa for MBCP2000 and MBCP. Mercury porosimetry gives 73% and 69% of total porosity respectively. The interconnections are evidenced by 3D reconstruction using qualitative and

quantitative microscanner. Permeability was twice higher for MBCP2000, and after incubation with bovine serum, 30% absorption increased with the MBCP2000 was observed. The low difference of total porosity between the 2 types cannot explain higher permeability, the performance is due to distribution of pore size particularly mesopores. The HA/TCP ratio was 20/80 for MBCP2000 and 60/40 for MBCP, and FTIR confirms high purity of HA and TCP without carbonate.

After implantation bone ingrowth is observed in the 2 types of bioceramic, and newly-formed bone progressively replaced the bioactive material, followed by haversian bone remodelling. Faster bone ingrowth into the macropores was observed at 6weeks for MBCP2000. After 12weeks no statistical difference was noticed between the 2 implants type. The rate of resorption however is higher for MBCP 2000: 17% versus 12% at 6weeks, and 19% versus 17% after 12weeks, (no significant difference).

The in vivo experiment indicated higher cell colonization by osteogenic cells in MBCP2000 due to this interconnected and microporous structure associated to higher solubility. However, due to bone ingrowth at the expense of the implant this phenomena is less evident after long term implantation. MBCP2000 is a more suitable matrice for tissue engineering. The HA/TCP ratio of 20/80 is also more efficient for combination with STEM cell cultivation and expansion then implanted in non bony site comparing to classical MBCP².

The kinetic of bone ingrowth by the osteogenic cells colonization need to develop inside the macropores. Without macropores and mesopores the bioactive processes are unable to develop in the deep of the implants. The association of dissolution at the crystal levels, the diffusion of the biological fluid into the micropores, and the resorption by macrophages and osteoclastic cells of the materials at the surface and inside the macropores, involve a progressive bone substitution of the materials by true bone. This is the common process of resorption/bone substitution of the Micro Macroporous Biphasic Calcium phosphate ceramics.

Conclusion

Advanced technologies for macroporous calcium phosphate bioceramics manufacturing involved higher efficacy of such matrices for further relevant surgical technologies as persurgery combination with bone marrow or expanded STEM cell in vitro for bone tissue engineering

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INSTRUCTIVE PROPERTIES OF BIOMATERIALS IN OSTEOCHONDRAL TISSUE ENGINEERING USING EMBRYONIC STEM CELLS

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Introduction

In previous studies with two bi-phasic calcium phosphate ceramics (BCPs) with different microporosity we observed opposite results when implanted intramuscularly in goats. The more microporous ceramic (BCP1150) was able to induce bone whereas the less micro porous (BCP1300) was not (Figure 1).

Mouse embryonic stem cells (mESCs) have the potential to differentiate into all adult cell types and can proliferate indefinitely. Moreover, they can easily be genetically manipulated and are donor independent and thus can serve as a model system for bone tissue engineering. MESC can be induced into the osteogenic lineage and where therefore assessed in this study as a model to investigate the molecular mechanism underlying the osteoinductive potential of ceramics.

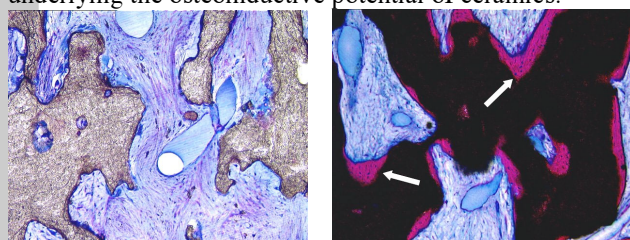


Figure 1: Histological sections of implanted samples in goat muscle. Left, BCP1300, no bone formation visible. Right, BCP1150, bone formation was present (arrows).

Methods

Embryoid bodies (EBs) were formed from IB10 mESCs, dissociated after 4 days and seeded onto 2x3x3 mm scaffolds of BCP1150 and BCP1300 and cultured in control and differentiation medium.

Results

We seeded mESCs on BCP1150 and BCP1300 scaffolds and assessed gene expression of osteocalcin and BSP, which were chosen as markers of mature osteoblasts. In all cases, differentiation medium enhanced expression of osteocalcin and BSP.

However the expression of osteocalcin and BSP was higher on BCP1150 than on BCP1300 reflecting the *in vivo* osteogenic behavior of BCP1150 (Figure 2A and data not shown). To investigate whether this was due to differential cell-material interaction or material-medium interaction we started by testing this last option.

Therefore we seeded cells onto BCP1300 in control and differentiation medium and added an empty scaffold of BCP1150 in the same well. The addition of an extra scaffold to the medium induces an increase in expression of both osteocalcin and BSP (Figure 2B), suggesting that

a change in medium composition by BCP1150 enhances gene expression of osteocalcin and BSP.

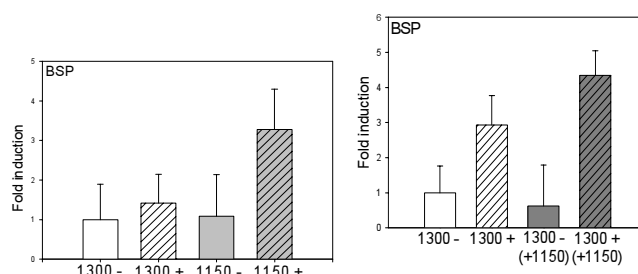


Figure 2: Differentiation of mouse embryonic stem cells. BSP expression of cells seeded on A. BCP1300, BCP1150 and B. BCP1300 with and without the addition of an empty BCP1150 scaffold (+1150), after 6 days of culture in control (-) and differentiation medium (+).

To investigate whether BCP1150 modifies the medium composition, we incubated medium with BCP1300 and BCP1150 and measured the medium components. We found a small decrease in calcium concentration in the medium after the addition of BCP1300 but a strong decrease after addition of BCP 1150 (Table 1). Medium incubated with both scaffolds, displayed a calcium decrease which was similar to the decrease of BCP1150. As such, the gene expression correlates to a decrease in calcium concentration (Figure 2A,B). In contrast to the calcium decrease the magnesium concentration was hardly effected.

Table 1: Calcium concentration in medium (M) treated with and without BCP1150 and BCP1300 scaffolds

Sample	Ca ²⁺ (µg/L)	Mg ²⁺ (µg/L)
M	75000	20000
M + BCP1300	70000	20000
M + BCP1150	55000	22000
M + BCP1300 + 1150	55000	22000

Discussion

In vitro, we found that mESC seeded on BCP1150 displayed enhanced gene expression of osteogenic markers compared to mESCs differentiated on BCP1300, which correlates with the *in vivo* data from the goat muscle implants. We also found that BCP1150 has an effect on the medium composition, whether this effect is caused by the decrease in the Ca²⁺ concentration is currently under investigation. In conclusion mESCs can be used as a cell source for testing osteogenic

ARTIFICIAL EXTRA-CELLULAR MATRICES BASED ON FIBRILS OF DIFFERENT COLLAGEN TYPES CONTAINING IMMOBILISED PROTEOGLYCAN (PGS) FOR TITANIUM IMPLANTS

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Introduction

Collagen in fibrillar form is used as a coating on titanium implants to mimic the natural bone extra-cellular matrix, as it presents binding sites for integrins and is not desorbed from titanium surfaces when placed in solution. Proteoglycans (PGs), consisting of a protein core connected to glycosaminoglycan chains, may be bound to these collagen fibrils during fibril formation. These in turn can influence osteoblast behaviour and also bind growth factors (GFs). The PGs decorin and biglycan, present in bone, are able to bind to and modulate the activity of the GFs TGF- β 1, which stimulates osteoblast proliferation and chemotaxis, and BMP-4, which enhances osteoblast differentiation [1]. The main aim of this work is to compare fibrils of the collagen types I, II and III with regard to amount of decorin and biglycan bound as well as the resulting changes in fibril formation kinetics and morphology. The reaction of primary osteoblasts and an osteoblastic cell line to titanium surfaces coated with fibrils containing decorin and biglycan was also studied, as was the effect of TGF- β 1.

Materials and Methods

Collagen types I (calf skin), II (bovine tracheal cartilage) and III (human placenta) as well as decorin and biglycan (bovine articular cartilage) were obtained from Sigma-Aldrich Chemie GmbH, Germany. Fibrillogenesis took place at 37°C in a 30 mM phosphate buffer at pH 7.4 at different PG:collagen ratios. Fibril mass was determined by the Lowry protein assay. PG content of fibrils was determined colorimetrically by hexosamine and dimethylmethylene blue assays. Fibril morphology was studied by atomic force microscopy (AFM). The kinetics of fibril formation were studied by means of turbidity measurements. Titanium surfaces were coated with fibrils and in some cases also with TGF- β 1 by adsorption, and seeded with an osteoblastic cell line (7F2), or primary rat calvaria (RCO) or human (HO) osteoblasts. Proliferation and collagen synthesis of RCOs and HOs were determined. 7F2 cells were tested for proliferation, calcium accumulation and alkaline phosphatase (ALP) production.

Results and Discussion

The results of both colorimetric assays correlated well. Collagen type II fibrils bound more PGs than fibrils of types I and III. Considerably more biglycan than decorin

was bound by all collagen types (Fig. 1). This could be due to biglycan's higher molecular weight and longer glycosaminoglycan chains, which may form more stable bonds with collagen by "bridging" more binding sites on collagen. Type II formed fibrils approximately half as thick as type I fibrils. Decorin caused fibrils of types I-III to become thinner, but biglycan had no effect on morphology. Morphological data was supported by kinetic data, which showed deceleration of fibril formation and reduction of turbidity by decorin but not biglycan. Type II may bind more PGs than type I because of its larger relative surface area due to its thinner fibrils. Type II may also have a higher inherent affinity for PGs than types I and III. HO proliferation and RCO collagen synthesis were modulated by the presence of both decorin and biglycan. TGF- β 1 modulated the proliferation and collagen synthesis of HOs. PGs did not affect 7F2 behaviour.

Conclusions

The ability of collagen fibrils to bind PGs varies according to PG and collagen type. Biglycan and Decorin influence fibrillogenesis kinetics and fibril morphology differently, and both PGs and TGF- β 1 modulate primary osteoblast behaviour on titanium surfaces coated with collagen fibrils. These results may be of importance when designing collagen-based extra-cellular matrices, including those loaded with GFs.

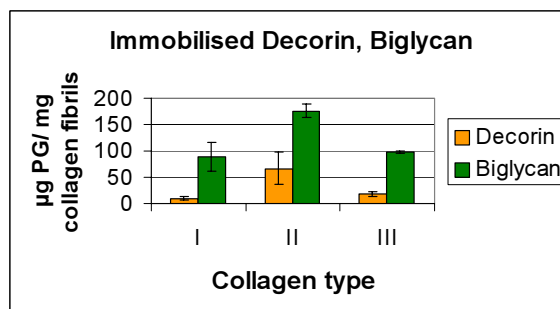


Figure 1: Quantification of PGs immobilised per mg collagen fibrils of collagen types I, II and III.

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BMBF, Germany for funding and T. Hanke for advice.

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MAGNETIC NANOPARTICLE-BASED TAGGING OF MECHANOSENSORS FOR BONE TISSUE ENGINEERING

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A major component of constructing connective tissues for regeneration and repair is the control of cell proliferation and activity. Mechanosensors in membranes are key regulators in the differentiation, proliferation and activity of bone cells. Activation and regulation of these mechanosensors has been proposed as a means by which engineering of tissues may be enhanced. Generating functional bone and connective tissue *in vitro* relies on culture environments which condition the tissue prior to implantation. Multiple protocols for the use of bioreactors which exert forces such as fluid flow and compression have been proposed. In this presentation, we describe a different approach where we target specific mechanosensors directly on human bone cell membranes within 3D constructs. By controlling the mechanical environment of the cells within the construct, we are no longer reliant on using strong materials which are capable of withstanding significant loading for bone tissue engineering. Hence, we aim to increase the turnover relationship between matrix and more rapidly degrading scaffolds in response to mechanical stimulation.

Using a magnetic force bioreactor developed in our lab, we describe our investigations into the internalization of magnetic nanoparticles which can bind to receptor sites on the internal membrane. By applying time-varying magnetic fields, we can generate forces on these receptors which result in downstream changes in gene expression and enhanced matrix production. A comparison of data generated from the type of receptor tagged such as integrins, ion channels and growth factor receptor sites will be described. In addition, this technique can be applied to human mesenchymal stem cells (Poetics, Ltd) in monolayer culture demonstrating an upregulation of differentiation markers such as osterix, cbfa1 after 1 week of cyclical loading in culture. The magnetic nanoparticle technology has the potential to be applied directly *in vivo* in animal models and ultimately in clinical treatments. We describe recent investigations into localisation of stem cells *in vivo* using magnetic tagging and tagging of key receptors for manipulation.

NEW APPROACH TO MICROPATTERNING FOR BIOMEDICAL USE

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Introduction

Micropatterning of adherent cells is a powerful experimental tool for studying the effect of cell shape on cell functions [1]. Despite the fact that photolithographic methods evolved a lot, treatment technique for large three-dimensional biomaterials implants such as the inside surface of an artery graft is still inexistent. Moreover the geometric resolution these methods achieve is useless in the context of patterning large biomaterials. A new technique of micro patterning is thus presented.

Materials

An air atomizing nozzle (Spraying Systems Co., Wheaton, IL) was mounted on a goniometer rail. The volume and flow rate of the solution were controlled using a Cole-Parmer pump (Vernon Hills, IL). A regulator controls the air pressure. A medical grade needle (23 gauge, 38 mm length) with the sharp end cut straight and polished was plugged in a 2.5 cc Hamilton syringe and fitted through the fluid cap. A microscope slides holder was mounted on the rail. The characterization of the surfaces was made using image analysis software (CLEMEX Vision, Longueuil, Qc).

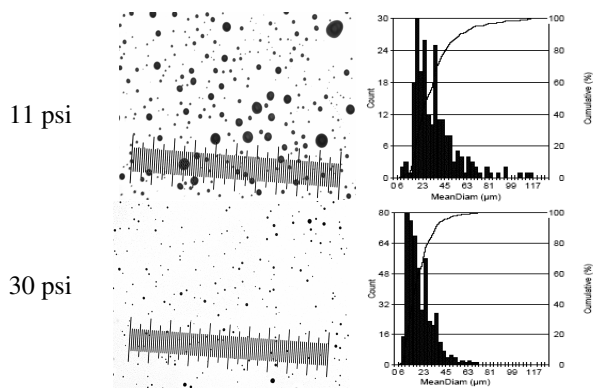


Figure 1: Surface image at 11 and 30 psi and their associated plot obtained with the CLEMEX vision software analysis.

Patterns were first characterized using aqueous solutions of glycerine (7% v/v), colored with blue dye and sprayed directly on the glass slides. Patterns of peptides were made by spraying a 1:1 mixture of CGRGDS and CWQPPRARI in a 1 μ M/ml solution on PTFE samples [2]. Proliferation tests were made using human umbilical vascular endothelial cells (HUVECs)

incubated at 37°C in culture media. The cells proliferation, adhesion and morphology were assessed by immuno fluorescence (DAPI and rhodamine-phalloidine).

Results and Discussion

The technique permits to pattern large areas with round spots of predetermined mean diameter and density. Air pressure and volume of liquid sprayed were sufficient to obtain a control on the patterns characteristics. We demonstrated that human endothelial cells grown on PTFE samples patterned with the aforementioned method had a modified adhesion and growth behaviours. The results show the possibility to control parameters of the patterns such the distribution and the average diameters of the spots.

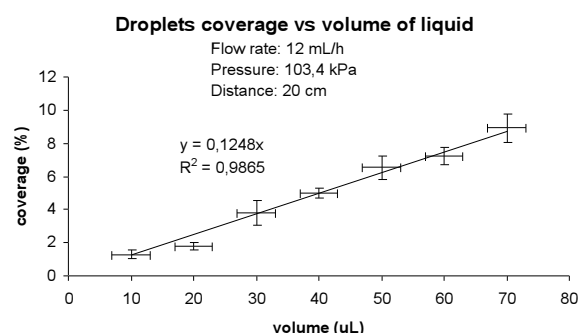


Figure 2: Effect of air pressure on surface coverage.

Conclusion

This method is an innovative, low costs, rapid, and simple method. The method exploits the semi-random characteristics of aerosols and the principles of liquids atomisation to imitate natural patterns.

Acknowledgements

Maude Larouche for technical support

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BIOINTERFACES DIRECTING CELL AND HOST RESPONSES

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Introduction

Cell adhesion to adsorbed ECM proteins and adhesive sequences engineered on synthetic surfaces plays critical roles in biomaterial, tissue engineering, and biotechnological applications [1]. Cell adhesion to these adhesive motifs is primarily mediated by integrin receptors. In addition to anchoring cells, integrins activate signaling pathways regulating cell survival, proliferation, and differentiation. While tethering short peptides derived from ECM ligands [e.g., RGD for fibronectin (FN)] promotes cell adhesion and function in several systems, these strategies are limited by reduced biological activity compared to the native ligand, lack of specificity among integrins, and inability to bind non-RGD integrins. We have engineered biointerfaces that mimic the secondary and tertiary protein structure of fibronectin and type I collagen. These surfaces convey integrin binding specificity and signaling as well as bone and muscle cell adhesion, proliferation, and differentiation. Furthermore, these interfaces enhance in vivo responses to implanted devices.

Materials

The collagen-mimetic, triple helical GFOGER peptide [GGYGGGPC(GPP)₅GFOGER(GPP)₅GPC] was synthesized via tBoc solid-phase chemistry [2]. The fibronectin-mimetic ligand was a recombinant fragment of FN (FN7-10) that encompasses the PHSRN and RGD binding domains from the native protein that synergize to specifically target the $\alpha 5 \beta 1$ integrin [3]. Bioadhesive ligands were tethered onto protein-adsorption-resistant supports (19:1 tri(ethylene glycol)-terminated/COOH-hexa(ethylene glycol)-terminated alkanethiol monolayers on Au) to target specific integrin adhesion receptors. Cell adhesion was analyzed in terms of integrin binding, focal adhesion assembly and signaling, and adhesion strength using biochemical and functional assays. Cell differentiation (gene and protein expression, mineralization) was assessed via real-time RT-PCR, immunostaining, and histochemical staining. For implantation studies, commercially pure titanium rods were coated with saturating densities of bioadhesive ligands and evaluated in a rat proximal tibia implantation model [4]. Osseointegration at 4 weeks was assessed by histomorphometry and mechanical testing.

Results

Controlled densities of tethered bioadhesive ligands were obtained by varying the coating solution concentration. Cell adhesion exhibited ligand density-dependent increases for both GFOGER- and FN7-10-functionalized surfaces. No adhesion was detectable on control (no

ligand) surfaces. Adhesion to GFOGER- and FN7-10-engineered surfaces was primarily mediated by $\alpha 2 \beta 1$ and $\alpha 5 \beta 1$ integrins, respectively, as demonstrated by blocking antibodies. Bioadhesive surfaces also promoted focal adhesion assembly and FAK activation. Furthermore, bioadhesive surfaces enhanced osteoblast proliferation, differentiation, and matrix mineralization compared to unmodified controls and surfaces modified with RGD peptide. Implantation analyses revealed that GFOGER-coated titanium implants enhanced bone-implant contact area (63%, $p < 0.01$) and improved implant anchorage (50%, $p < 0.000001$) compared to unmodified control titanium (Figure 1).

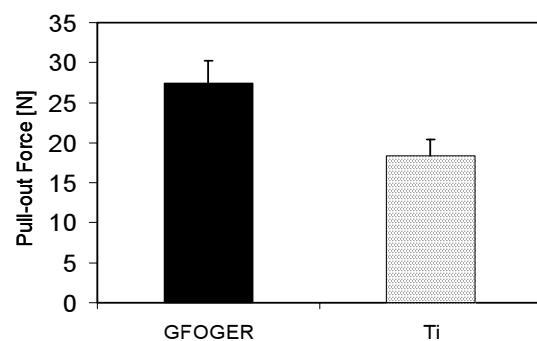


Figure 1: Pull-out force for titanium rods implanted in the rat proximal tibia for 4 weeks.

Discussion and Conclusions

We demonstrate that presentation of the collagen-mimetic peptide GFOGER and fibronectin-mimetic recombinant fragment containing the RGD and PHSRN motifs results in integrin-specific adhesion and signalling and promotes osteoblast proliferation and differentiation. Moreover, coating GFOGER on titanium implants enhanced osseointegration and mechanical fixation in an in vivo model. These results show that biomolecular strategies focusing on integrin binding specificity improve biological responses to implants.

Acknowledgements

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TRACTION FORCE MICROSCOPY OF CARDIOVASCULAR CELLS

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Introduction

Cells of the cardiovascular system, such as endothelial cells and neutrophils, are required for hemostasis. Endothelial cells are the main cellular component of blood vessels, and neutrophils are the first cells required for the cellular inflammatory response. Endothelial cell-cell interactions are important for the formation of blood cells during angiogenesis. Neutrophils are highly motile cells that exert weak forces but crawl quickly. Thus, the actions of both types of cells require mechanical interactions. Traction force microscopy is a technique that allows imaging of the forces that cells exert on surfaces as they are adhering and spreading. Here we use traction force microscopy to describe the adhesion and spreading of endothelial cells, the interactions among endothelial cells, and the forces exerted by neutrophils during motility.

Materials

Traction force microscopy (TFM) was conducted on polyacrylamide gels that were functionalized with adhesion ligands (RGD for endothelial cells and intercellular adhesion molecule-1, ICAM-1, for neutrophils). In TFM, beads embedded in gels are imaged along with cells to elucidate the forces that cells exert on surfaces as they are adhering. Calculation of the forces is conducted using an inverse Greens function method. Bovine aortic endothelial cells were deposited and spread on PA gels for periods of several hours and imaged by filtering light that caused cell damage. The repeated interaction among endothelial cells was imaged as a function of ligand density and gel compliance, which was altered by changing the crosslinking density. Neutrophils were harvested from whole blood and plated on gels containing ICAM-1. Neutrophils would spread and exert forces in response to the peptide f-m-l-p. Either uniform a concentration (chemokinesis) or gradients (chemotaxis) were applied to the cells, and traction images were harvested over up to 15 minutes of examination of cell tracking.

Results

1. Bovine Aortic Endothelial Cells adhere and spread on RGD-PA surfaces, as previously described¹. The contact area and the force of adhesion increase linearly with time. At low cell densities, cell-cell interactions are apparent through repeated cell-cell touching. The

character of cell-cell interactions was a function of the density of adhesion ligands on the surface. At low densities of RGD, cells repeatedly round up after touching, either pushed or pulled by neighboring cell types. At high densities of RGD, cells repeatedly touch but never form a lasting connection. The displacement of beads in the intervening gap between cells clearly shows that there are durotactic forces that allow two cells to communicate through the substrate. Further, measurements of the mean squared displacement between cells shows that durotaxis is more likely on compliant surfaces, since the motility shows evidence of hindered diffusion.

2. Neutrophils are fast moving cells that were thought to exert weak stresses and therefore couldn't be imaged in TFM. We show that when the gel compliance is appropriately set, force tractions in neutrophils can be imaged. We found that the largest stresses in the neutrophils are in the uropod, and that these stresses are counter-oppositional to the direction of motion. Further, we find that during a turn, neutrophils will reposition the direction of the uropod *before* the cells will turn in a new direction. A measure of the root mean square force generation in neutrophils shows that neutrophils exert substantial higher stresses during chemotaxis than chemokinesis, with a mean chemokinetic force of 34 pN.

Discussion

The differential adhesion hypothesis indicates that cell-cell assembly should be driven by cell-substrate adhesion and substrate compliance. Despite the presences of VE-cadherin, BAECs do not form lasting connections. We are examining whether other endothelial cells types do so.

It was previously thought that neutrophils could not be measured in TFM, but that is not true. Their motility is exactly the opposite of what is seen for most other motile cells, with forces first generated at the rear of the cell.

Acknowledgements

We wish to acknowledge support from the NIH-NHLBI and the Whitaker Foundation.

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MOLECULAR MECHANISMS DRIVING CHONDROGENIC DIFFERENTIATION OF ADULT STEM CELLS

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Introduction

Chondrogenic differentiation of human mesenchymal stem cells (hMSCs) offers great therapeutic potential for the treatment of articular cartilage defects. At present, *in vitro* chondrogenesis is driven by empirically derived protocols comprising a high-density cell aggregate culture system¹. The Notch pathway is a highly conserved signalling mechanism involved in many processes determining cell fate during development. In this study we analysed the Notch signalling pathway during chondrogenic differentiation of human mesenchymal stem cells.

Materials and Methods

hMSCs from bone marrow (Cambrex) were expanded to passage 4 in the presence of FGF-2, (5ng/ml). hMSCs were placed into 3D cell aggregate culture, (500,000 cells /cell aggregate)¹ for up to 14 days. Real-time qPCR using Sybr Green I was carried out to analyse the gene expression profiles of the four Notch receptors, their five ligands and downstream effectors HES-1 and HEY-1. Jagged-1 protein expression and localisation was analysed by immunohistochemistry of 3D cell aggregates. Full length Jagged-1 cDNA was generated from human placental RNA to produce recombinant Jagged-1 adenovirus (Ad-Jag-1). hMSCs were transduced with either Ad-Jag-1 or with a control adenoviral GFP (Ad-GFP). Post-transduction, hMSCs were placed into chondrogenic cell aggregate culture. Notch activation was assessed by qPCR of HEY-1 gene expression. Chondrogenesis was assessed by qPCR analysis the known chondrocyte markers collagen II and aggrecan and by histological and immunohistochemical characterization at 14 days.

Results

Initiation of chondrogenesis by 3D cell aggregate culture was accompanied by a general down-regulation of Notch receptors. Jagged-1 was the only Notch ligand expressed and it increased transiently by 3-fold to a peak at day 2 ($P<0.05$) (figure 1A), this was concurrent Jagged-1 protein was immunolocalised only within regions that subsequently showed chondrogenic differentiation. Transduction of hMSCs with Ad-Jag-1 caused a 45-fold increase in Jagged-1 message level ($P<0.05$) over Ad-GFP control hMSCs, this level of expression was maintained throughout 14 days of 3D cell aggregate culture. qPCR analysis for HEY-1² revealed that the increased expression of Jagged-1 resulted in continuous Notch signalling throughout the culture period. Over-expression of Jagged-1 ligand and the subsequent persistence of Notch signaling over 14 days resulted in complete inhibition of chondrogenesis. Ad-Jag-1 cell aggregates were smaller than the Ad-GFP controls; cells did not acquire a chondrocyte-

morphology and failed to deposit a GAG-rich matrix as displayed by safranin O stain (figure 1B). Collagen II immunolabelling was also negative and collagen II and aggrecan gene expression were 1.5% and 15% that of the Ad-GFP controls at day 14 respectively ($P<0.05$).

Discussion

Notch signalling was active transiently during the early stages of chondrogenesis (days 1-4). The transient rise in Jagged-1 was also strongly suggestive of an active role for Notch signalling in chondrogenesis. The sustained expression of HEY-1 in Ad-Jag-1 transduced cells in cell aggregate culture showed sustained Notch signalling, and hence suggested that Notch signalling must be stopped for chondrogenesis to proceed. Jagged-1 expression on osteoblasts in bone marrow is has been suggested to maintain the self-renewal of haematopoietic stem cells and prevent further differentiation. Therefore, Notch signalling amongst mesenchymal stem cells may retain them undifferentiated and transient Notch in cell aggregates may prime and synchronise chondrogenesis in adult stem cells.

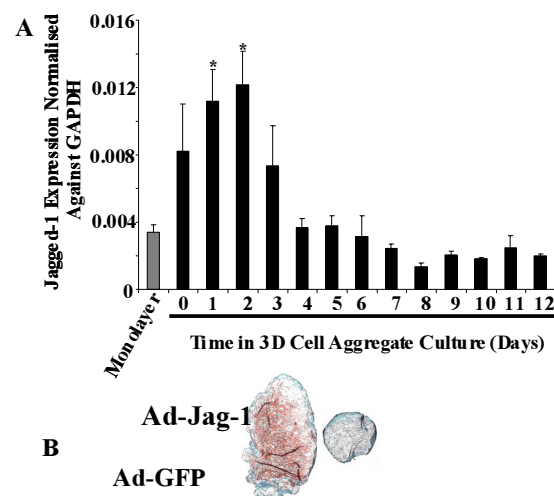


Figure 1: (A) Jagged-1 gene expression in hMSCs during cell aggregate culture. * = $P<0.05$ compared to monolayer expression levels. (B) Safranin O stain of Ad-GFP and Ad-Jag-1 cell aggregates at day 14.

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Acknowledgements

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GRAFTING OF CYCLODEXTRINS ON PET VASCULAR PROSTHESES FOR CONTROLLED RELEASE OF ANTIBIOTICS

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Introduction

The most currently used vascular prostheses are made of polyester (PET) woven yarn (Dacron® fibre). They are biocompatible, but implantation induces post-operative infections in 6% of the clinical cases [1]. Cyclodextrins (CDs) have the ability to form "inclusion complexes" with a wide variety of drugs. It is possible to graft CDs onto textile supports, and we established the concept of a vascular prosthesis modified with CDs, adsorbing antibiotics, and able to release them within a prolonged period preventing the post operative infections.

Materials and Methods

Different CDs (β -CD, γ -CD) and Hydroxypropyl-CDs (HP β -CD, HP γ -CD) were grafted onto vascular PET prostheses [2]. The optimal fixation temperature and time of curing for each type of CD was determined. The finishing reaction did not induce any alteration of the mechanical properties. In addition, it contributed to their blood tightness. Multiple tests on cytocompatibility, hemocompatibility and antibiotic releasing were performed.

Results

Biological evaluation by cell viability tests confirmed the non-toxicity of grafted surfaces. Although cell vitality assessments show clear cell activation, proliferation tests revealed a low adaptation of epithelial and endothelial cells (L132 and HPMEC) (Fig. 1) due to the woven and rough structure of the grafted prostheses (Fig. 2). This has also been shown for both cell types by cell adhesion kinetics and catechetical staining of the cytoskeleton (actin and vinculin).

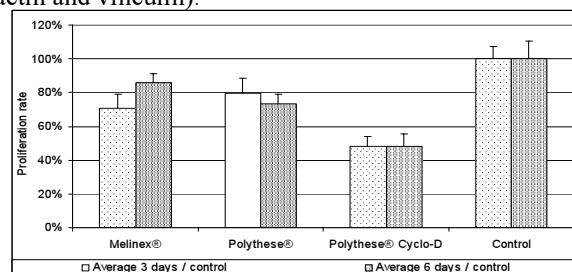


Figure 1: Proliferation rates of HPMEC on pure PET (Melinex®) and on virgin and grafted prostheses with HP γ -CD (Polythese) (n=6).

After loading the CDs with different antibiotics, their *in vitro* release was assessed by using UV spectrophotometry. It revealed their presence in water up to 30 days, and in plasma up to 72 hours. Ungrafted woven PET released the antibiotics immediately.

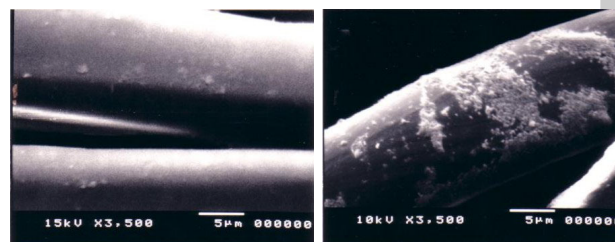


Figure 2: SEM micrographs of virgin (left) and HP β -CD grafted woven PET (right).

Bacteriological tests showed that grafted PET inhibited growth of *S. aureus* for 72 hours with rifampicine and 24 hours with ciprofloxacin and vancomycine in plasma, when this was renewed during the experiments all 2 hours (Fig. 3). Compared to virgin or collagen coated prostheses, CDs promote a significantly prolonged antibacterial effect, with respect to prostheses not grafted with CDs.

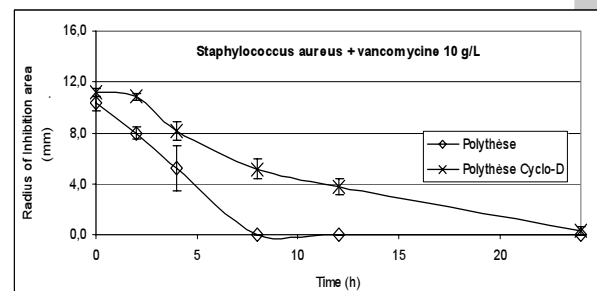


Figure 3: Prolonged antibiotic effect of released Vancomycine from HP β -CD grafted woven PET.

The nine different standard hemocompatibility tests (ISO 10993-4) revealed a perfect blood compatibility under static and dynamic conditions.

After selection of the HP γ -CD by multiple exclusion criteria, the final prosthesis is now in animal experiments.

Acknowledgements

The authors gratefully acknowledge the contributions of CJ Kirkpatrick, U Seyfert and Laboratoires Pérouse.

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BIOMATERIALS DISCOVERY USING COMBINATORIAL SYNTHESIS AND COMPUTATIONAL MODELING

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Introduction

Combinatorial synthesis followed by high-throughput screening and computational modelling of the data has been applied successfully in several materials discovery projects.¹ But, the use of such techniques in the development of biomaterials is still new and poses several unique challenges. In particular, standard performance metrics for in-vitro biomaterials evaluation must be found and validated. One such metric is protein adsorption. Adsorption of various proteins to surfaces is known to be critical in cell attachment and, therefore, is a major determinant of the suitability of materials for medical-implant applications. In particular, the protein fibrinogen is known to participate in processes leading to blood clotting. Thus, fibrinogen adsorption can have an adverse effect on the performance of blood contacting implants. Alternatively, one can measure more directly the response of cells to a synthetic surface. A variety of available cell assays allows the study of several different types of cellular responses including proliferation, genotypic expression, migration, or differentiation.

We have used combinatorial synthesis in the development of tyrosine-derived biomaterials for medical applications.² The goal is accelerate the discovery process for new biomaterials. Thus, it is necessary to establish correlations between experimental results and calculated polymer properties that can be used to refine the synthesis process. As we show in the current work, this can be done using an arsenal of computational methods. These methods, well established in other fields of research, are now adapted by us to biomaterials.

Materials

The methods used for synthesis, characterization, and computational analysis have been published before.³⁻⁶

Results and Discussion

The laboratory of Kohn has used tyrosine-derived monomers to prepare a wide range of polymers including the first combinatorially designed library of polyarylates. In this library, 14 different tyrosine-derived monomers and 8 different dicarboxylic acids were copolymerized in all possible combinations, forming a library of 112 distinct, strictly alternating A-B copolymers. In the course of the characterization of the 112 polymers, data sets with hundreds of individual data points were obtained. To illustrate the methodology, we focus here on the study of protein-polymer interactions. Specifically, we screened a selected set of 46 polymers for polymer-adsorbed human fibrinogen using our immunofluorescence assay. We also measured cell growth on 46 randomly selected polymer surfaces under standard cell growth conditions. Next, semi-empirical Artificial Neural Network (ANN) Models were developed to

predict fibrinogen adsorption and fetal rat lung fibroblast growth. These models were then asked to predict the amount of protein adsorption and the rate of cell growth on previously untested polymer surfaces. ANN predictions of fibrinogen adsorption were correct for 39 of the 46 test polymers, while ANN predictions of cell growth were correct for 42 out of 48 polymers to within the average percent experimental error.

While these results demonstrate the utility of fairly simple computational models to predict complex biological phenomena (such as cell growth), this work still followed the traditional approach of synthesis first, followed by modelling. Synthesis is needed first because at least some experimental data is needed to train the ANN models. In recent work, molecular dynamics simulations of the hydrated polymer surfaces were used to compute a radial distribution function (RDF) for the distribution of water around specific polymer functional groups. These RDF's were then applied as inputs into the ANN. Likewise, a wide range of polymer descriptors can be calculated directly from the polymer structure. Using these advanced techniques, it was possible to predict fibrinogen adsorption with very high accuracy *without any prior experimental data*.

Conclusions

The use of MD simulations to calculate molecular descriptors for ANN models is a promising approach for the prediction of biomaterials properties prior to their synthesis. This approach opens the door for the computational discovery of lead polymers in large polymer libraries.

Acknowledgements

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MULTIFUNCTIONAL POLYMERS FOR BIOMEDICAL APPLICATIONS

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Most polymers used in medical applications today are materials that have been developed originally for application areas other than biomedicine. Considering this, different biomedical applications are demanding different specifications for the properties and the functionalities of the biomaterials. Compared to the intrinsic material properties, we define functionality as a certain combination of the polymer architecture and a specific process. Examples for functionalities that play a prominent role in the development of multifunctional polymers for medical applications are biofunctionality, degradability, or shape-memory functionality. In this sense, multifunctional polymers are tailor-made for specific biomedical applications. This concerns not one single synthetic material, but polymer systems whose macroscopic properties can be tailored over a wide range by variation of molecular parameters.

Shape-memory signifies the ability to trigger predefined shape changes by external stimuli. Thermally-, light-, and magnetically induced shape-memory polymers will be presented, that were developed especially for minimally invasive surgery and other biomedical applications. An example for an application is an intelligent suture material that increases stress in a predefined mode, actually tested in preclinical studies.

Active scaffold structures as temporary substitute of the complex natural extracellular matrix are another example for applications of such multifunctional materials. The polymer scaffolds serve as temporary substrate and substitute of the complex natural extracellular matrix in order to provide a suitable environment for tissue formation

Modified polyetherimide (PEI) materials were found to be non-cytotoxic according ISO10993-5. The biocompatibility was evaluated by cell culture experiments of human fibroblasts in direct contact with modified PEI with respect to cell proliferation, morphology and

function. It is especially well suited for applications in the medical area because of its membrane forming properties and its thermostability, i.e. it is steam sterilisable. Polyetherimide can be modified easily with nucleophiles. In the wet-chemical treatment of polyimide membranes with aminic modifiers, the modifier molecules will be covalently bound to the membrane polymer. When using modifiers with high aminic nitrogen, the amination is combined with a degradation process which shifts separation properties from ultrafiltration characteristics (untreated membrane) to microfiltration characteristics (treated membrane).

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THE INFLUENCE OF IMPLANT SURFACE TOPOGRAPHY OVER CHEMISTRY

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Introduction

Orthopaedic implant finishes vary from electropolishing of stainless steel (SS) to micro-rough titanium (TS) and Ti-6Al-7Nb (NS). In the context of soft tissue, represented *in vitro* by fibroblasts, rough versus smooth on TS and SS did not significantly affect cell adhesion or subsequent growth. However, fibroblast spreading and cell growth were seriously compromised on micro-rough NS [1]. This led to the question; is the contributory cause chemistry or topography or a combination of both? Here we investigate the influence of chemistry and topography by means of surface coating and micro-fabrication.

Materials

Surface chemistry was masked by evaporating 50nm of either gold or titanium on NS, TS and SS. Surfaces were characterised with Scanning Electron Microscopy (SEM) and X-ray Photoelectron Spectroscopy (XPS). Qualitative assessment utilised SEM for cell growth and morphology at 24h, 5 and 10 day timepoints and Fluorescence Microscope (FM) for quantitative cell counts. Intracellular components, vinculin, tubulin, actin and DNA, were fluorescently labelled and imaged using the FM at 48h, with vinculin labelled focal adhesion (FA) sites counted and measured. A microtopography die was produced utilising photolithography and wet etching methods. Replicas were produced by embossing in polycaprolactone and sputtering with 50nm titanium.

Results and Discussion

Surface coating of NS produced a homogenous masking layer of either gold or titanium, confirmed by XPS and SEM. Cell growth demonstrated no statistically significant differences per sample (coated or uncoated NS) type at 24h, 5 or 10 days – cell growth was typically depressed on all sample types of NS. To safeguard against coating compatibility issues, SS and TS were also coated with gold or titanium and cell growth was demonstrated to be normal for these surfaces in comparison with the uncoated versions – confluent monolayers were observed at 10 days on all, and confirmed quantitatively. These results indicate that for NS, the surface topography and not the underlying chemistry was the primary cause of inhibited cell growth. This finding was confirmed with coated SS and TS surface models.

While NS and TS demonstrated similar roughness averages of 0.77µm and 0.90µm respectively, morphologically these surfaces differed with NS displaying a rough microspiked topography. These - phase microspikes, a characteristic of Ti-6Al-7Nb duplex alloy, were demonstrated by AFM to have

surprisingly uniform dimensions. They were also demonstrated by FM to interfere with cellular processes of adhesion and microtubule formation, and hypothesised to be the primary surface attribute for the observed suppression in cell growth.

Inspired by the dimensions and general morphology of the NS microspikes, a metallic pyramid topography was fabricated. Cells on these topographies were demonstrated to display low cell growth, low spreading, and their adhesion sites were visibly influenced by the presence of the pyramids. Quantitatively, the adhesion site (FA) numbers and mean lengths were observed to be similar to those measured on NS, and both considerably lower than a planar control surface.

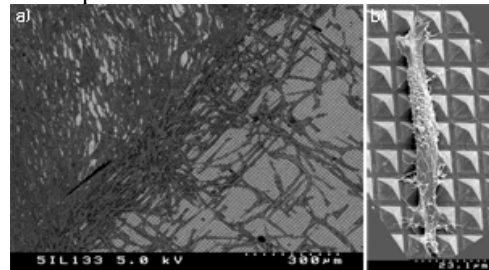


Figure 1: (a) Cells cultured for 10 days at the pyramid border. (b) 'Typical' cell morphology on pyramid microtopography.

Conclusions

The masking of heterogeneous surface chemistries enables biological comparisons to be made of numerous different material topographies, independent of their original elemental composition. In this instance, surface topography, and indeed morphology, proved to be a far more important factor. This is further illustrated by the microfabrication approach, in which mimicking particular significant surface morphology elicits similar cellular reactions. It is anticipated that this novel experimental approach might aid in future developments of metal implant topography for optimisation of both soft and hard tissue integration to the implant. A reciprocal trend could be developed, as metal topographies could provide inspiration for fabricated microtopographies that in turn could be utilised to produce optimal metallic implant topographies, and not only with regard to fibroblasts.

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INCREASED CYTOSKELETAL DYNAMICS OF INVASIVE VS. NON-INVASIVE TUMOR CELLS

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Introduction

Different tumor cell lines as well as individual tumor cells have different capacities to metastasize. Metastasis formation involves tumor cell transmigration through the endothelium and invasion into tissue. Here we tested the hypothesis that invasive tumor cells have a highly dynamic cytoskeleton which allows them to change their shape or reorganize focal adhesions more rapidly than non-invasive cells.

Materials

We measured the invasiveness of nine tumor cell lines isolated from different organs (bladder, breast, cervix, colon, kidney and lung) in a 3D collagen gel assay. Cells were plated on the gel surface and incubated for 3 days. The number density of tumor cells that invaded into the collagen gel times their average invasion depth was taken as an index of invasiveness.

Cytoskeletal dynamics of the tumor cells were measured using magnetic tweezer microrheology. Step forces between 0.3 nN and 3 nN were applied to fibronectin-coated superparamagnetic beads bound to the cytoskeleton of adherent tumor cells. Bead displacement in response to the applied force followed a power law. The power law exponent was taken as a measure of cytoskeletal dynamics, with low values corresponding to a solid-like, static behavior, and high values corresponding to a more liquid-like, dynamic behavior (1).

Results and Discussion

Six tumor cell lines were found to be invasive. Invasive tumor cell lines had a significantly higher power-law exponent (0.34 ± 0.03) than non-invasive tumor cells (0.26 ± 0.02 , $p < 0.01$).

To exclude effects of different receptor expression levels and bead internalization among tumor cell lines, we coated the beads with an antibody directed against $\alpha\beta 5$ integrin which was expressed at similar levels in all tumor cell lines. Measurements on invasive MDA-MB-231 and non-invasive MCF-7 tumor cell lines showed no difference between invasive and non-invasive cells.

Conclusions

These results indicate that the $\alpha\beta 3$ integrin and other fibronectin-receptors but not $\alpha\beta 5$ integrins play a role in cytoskeletal remodeling processes. Further, these findings are consistent with the hypothesis that tumor cell invasiveness correlates with cytoskeletal dynamics. Microrheology measurements may help identify molecules and signal transduction pathways that control cytoskeletal dynamics and hence tumor cell metastasis.

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EFFECTS OF EXOGENOUS PROSTAGLANDIN E₂ ON HUMAN MESENCHYMAL STEM CELLS

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Introduction

Human mesenchymal stem cells (MSC's) have the proven ability to differentiate into various cells types like chondroblasts, osteoblasts, adipocytes, fibroblasts and muscle cells. The differentiation process takes place dependent on the culturing medium containing the promoting factors.

In our case the glucocorticoid dexamethason (dex) which blocks the cyclooxygenase 2 (Cox2, inducible Cox) is the factor that drives the MSC's to undergo differentiation to osteoblasts.

Prostaglandin E₂ (PGE₂) produced by Cox2 is supposedly a critical regulator of bone metabolism shown in rat calvarial cells [1] and mouse osteoblastic cell lines[2]. Whether it has such a function on differentiating human MSC's to osteoblastic lineage we tried to show in this study.

Material

The MSC's were collected from bone marrow donors and separated via gradient centrifugation.

The cells were plated on polystyrene wells at a density of 5×10^3 , trypsinized and cultured until 6th passage. Medium exchange was done twice a week with 1 μ M PGE₂, 10nM Dexamethason in (DMEM). Effects of PGE₂ on MSC were obtained by measuring different osteoblastic markers like alkaline phosphatase (ALP) and collagen synthesis after 11d or 15d of culture. Real-Time-PCR data showed the gene expression of early (cbfa1, ALP, Osteopontin) and late (Osteocalcin, Osteoprotegerin) markers of osteoblastogenesis. Immunofluorescence was used to visualize morphological changes in differentiation.

Results and Discussion

The parameters that distinguish differentiating osteoblasts from MSCs are a high activity of ALP, collagen production and increased gene expression of core binding factor α 1, Osteopontin, Osteocalcin, Osteoprotegerin and Collagen Ia, therefore lower levels of RANKL and PPAR γ expression.

The ALP activity was upregulated in the dex samples and even a higher yield with added PGE₂ is seen. In terms of gene expression the ALP was expressed at higher levels in the dex samples with slight decrease with exogenous PGE₂ but PGE₂ alone didn't enhance expression.

Cbfa1 as a major regulating transcription factor involved in bone metabolism is upregulated already after 11d as well as after 15d of treatment. Osteopontin mRNA levels showed no significant changes after 15d of culture but Osteocalcin mRNA content was increased after either treatment with dex, PGE₂ and dex/PGE₂. Osteoprotegerin as the secreted factor for enhancing osteogenesis is increased in all treated samples whereas RANKL as the osteoclast's attracting factor is kept at a low level Collagen Ia expression as well as the production is elevated in the samples treated with dex but RNA expression was also high in PGE₂ treated cells. The adipocyte marker PPAR γ showed an increasing level in either one treated sample and PGE₂ seems to enhance adipocyte gene expression in dex treated cells.

Phenotypic changes were seen in PGE₂ treated cells. They seem more compact with loss of focal contacts compared to untreated cells.

The interesting question behind these results arised when we compared non-treated cells with PGE₂ treated cells where no clear signals for the osteoblastic differentiation could be determined or when the comparison was done with decks treated and dex/PGE₂ treated cells to realize the signals which we are missing because of the cox2 inhibition by dex treatment.

Conclusion

In our case we saw that PGE₂ obviously does interfere with the dex signaling when pushing MSCs to the osteoblastic lineage. The enhancement of the ALP activity of decks treated cells through PGE₂ might eventually work by (re)activating the cox2 gene expression and further signalling steps which are primarily inhibited through dex.

PGE₂ does not interfere with collagen production shown in the same amount of collagen Ia.

The prostanoid PGE₂ is a factor that influences osteoblastic differentiation of MSCs. How strong this influence turns out to be and to what extend it is an osteoinductive factor is still to be determined.

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BONE TISSUE ENGINEERING - FROM CELLS, MICE TO MAN - BRIDGING THE GAP

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Introduction

Given the demographic challenges of an ageing population combined with rising patient expectation and the growing emphasis placed on cost containment by health-care providers, economic regenerative medicine approaches for skeletal regeneration is a major clinical and socio-economic need. Mesenchymal stem cells or human bone marrow stromal stem cells are defined as multipotent progenitor cells with the ability to generate cartilage, bone, muscle, tendon, ligament and fat. These primitive progenitors exist postnatally and exhibit stem cell characteristics, namely low incidence and extensive renewal potential. These properties in combination with their developmental plasticity have generated tremendous interest in the potential use of mesenchymal stem cells to replace damaged tissues [1]. To date, relatively little is known concerning the phenotypic characteristics, whether from a morphological or biochemical standpoint whilst direct *in vivo* confirmation of the lineage potential and plasticity or interconversion potential that exists of mesenchymal stem cells and osteogenic progenitor cells remains unclear.

Materials and Methods

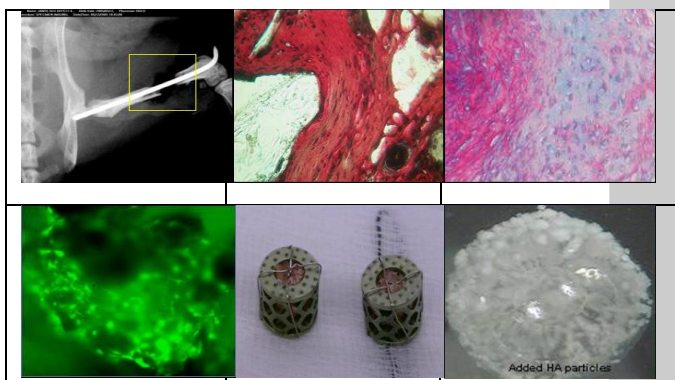
We have established protocols for the isolation of human fetal and adult mesenchymal populations using the monoclonal antibody STRO-1 and MACS and FACS strategies [2]. In addition much of our work is centred on the development of bone formation strategies using appropriate scaffolds (biomimetic polymers and biomimetic-inspired scaffolds based on chitosan and alginate and calcium carbonate) and judicious selection of growth factors as well as analysis using molecular strategies of mesenchymal differentiation and plasticity

Results

Current approaches and challenges from work in the Southampton group centered on: i) isolation, expansion and characterisation of the plasticity of fetal and adult osteoprogenitor populations tissue, ii) combination of osteoprogenitor cells with innovative self assembling biomimetic matrices and, iii) use of selected osteotropic agents and biomimetic scaffolds to modulate the phenotype of the mesenchymal stem cell to generate mineralised bone tissue.

Discussion

The biological understanding of stem cells is improving at an extraordinary rate but much greater developments in our understanding of mesenchymal stem cell and osteoprogenitor biology are of paramount importance if stem cell therapies are ever to become routine clinical



Cartilage formation using fetal mesenchymal populations and bone formation on PLA-BMP-2 encapsulated scaffolds. Top right shows an *in vivo* segmental defect model. Lower panels: Polysaccharide capsules containing hydroxyapatite inclusions for new bone formation, spinal cages for impaction grafting analysis and mesenchymal populations polymer scaffolds.

practice. Our initial clinical data, although very limited, is promising and offers much promise that the development of protocols, tools and above all multidisciplinary approaches for *de novo* bone formation that utilise mesenchymal stem may improve the quality of life for many as a result of strategies to augment skeletal regeneration.

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THE INTERCELLULAR CONTACT OF ENDOTHELIAL CELLS *IN VITRO* AS AN INDICATOR FOR BIOCOMPATIBILITY

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Introduction

Endothelial cells (EC) cover the inner surface of blood vessels and form the interface between the blood and the surrounding tissues. EC are involved in barrier function, which is regulated by the interendothelial cell contacts. These interendothelial cell contacts are functional units which are affected during (patho-)physiological processes such as inflammation and angiogenesis. Important molecules in the regulation of interendothelial contacts during the above mentioned processes are e.g. CD31, ZO-1, VE-cadherin, F-actin. They are activated in various ways by different soluble biological factors such as tumour necrosis factor α (TNF α) and vascular endothelial cell growth factor (VEGF).

In this study, pro-inflammatory and toxic stimuli and contact of endothelial cells to metallic surfaces were examined for their effects on the distribution pattern of interendothelial contact molecules.

Materials and Methods

Human dermal microvascular EC (HDMEC) were seeded in fibronectin-coated chamber slides and treated with pro-inflammatory (TNF α , endotoxin) and toxic stimuli (CoCl₂). Furthermore, EC were seeded on cpTi, and Co28Cr6Mo surfaces. After 24 and 48 h cells were fixed and stained for the above mentioned interendothelial contact molecules (immunofluorescence) and cell viability was analysed by MTS conversion assay.

Results and Discussion

EC *in vitro* show a specific pattern of the different interendothelial contact molecules (CD31, VE-cadherin, ZO-1) in that they are entirely distributed along the cell borders (Fig. 1a/b, CD31 and VE-cadherin in control cells). After treatment with different biological stimuli (TNF α , endotoxin) these molecules are distinctly redistributed and partially dissolved. This redistribution varies depending on the stimulus.

Toxic compounds (here: CoCl₂) also induce a redistribution of the interendothelial contact molecules (Fig. 1c/d, 24 h treatment, 0.7 mM). Here, the pattern of CD31-distribution is practically absent and the staining for VE-cadherin is faint after the CoCl₂-treatment. Interestingly, at this time point an MTS-test (indicating metabolic activity of cells) did not show any impairment with 0.7 mM CoCl₂ (Fig. 2). Higher concentrations (≥ 3 mM) showed a significant reduction of MTS-conversion. Extended exposure to CoCl₂ (3 days) showed impairment of viability also at 0.7 mM.

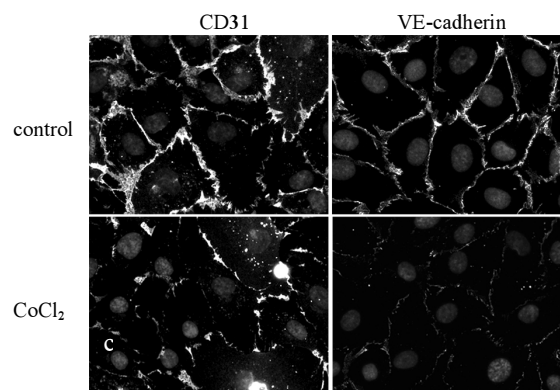


Figure 1: Staining for CD31 (a/c) and VE-cadherin (b/d) in HDMEC. a/b: untreated control c/d: CoCl₂-treatment

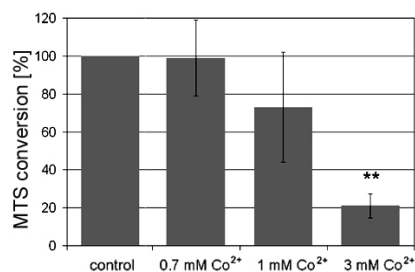


Figure 2: MTS conversion assay for HDMEC treated with different concentrations of CoCl₂ (24 h treatment).

Thus, the distribution of interendothelial contact molecules indicated a deleterious effect earlier than the classical cytotoxicity assay.

Direct contact of HDMEC to the different materials (i.e. cpTi and Co28Cr6Mo) also induced differences in the distribution of the interendothelial cell contact molecules. Thus, direct contact to Co28Cr6Mo-surfaces induced strong deviations (CD31 was redistributed, VE-cadherin and ZO-1 were hardly visible); contact to cpTi did not induce any marked alterations (data not shown).

Conclusions

We could show that the examination of interendothelial cell contacts is a sensitive indicator for a stressed endothelium. Thus, examining the pattern of interendothelial contact molecules in EC *in vitro* may be useful for testing EC-compatibility of biomaterials (e.g. stent materials).

Acknowledgements

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EXPERIMENT AND MODELLING OF PATTERN DEVELOPMENT DURING FIBRONECTIN NANOFIBRIL FORMATION

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Introduction

Cell-cell and cell-substrate binding are supported by extracellular matrix proteins like fibronectin. The formation of fibrils from single fibronectin molecules is a complex process which includes binding of cell receptors, like integrins, conformational changes of fibronectin by mechanical forces exerted from the cell, and polymerisation of fibronectin molecules at newly exposed binding sites. By using a graded physicochemistry of polymer substrates fibronectin fibrillogenesis of endothelial cells was studied in respect to its micrometer scale and nanometer scale pattern.

Methods and Results

Adherent endothelial cells reorganize fibronectin molecules in the extracellular space into an ordered fibrillar network with characteristic patterns on the microscale as well as on the nanoscale. Cell culture experiments on plane glass substrates coated with maleic anhydride copolymers exhibiting a graded physicochemistry yield a dependence of the fibronectin fibril pattern on the modulated anchorage strength of fibronectin to the substrates. The distinct spacing of paired fibronectin fibrils observed on the nanometer scale by scanning force microscopy (SFM) with its repeating unit of 71 nm could be correlated to the force sensitivity of the adhesion apparatus of the cell and the inner structure of the actin stress fibres with its crosslinker α -actinin as a structural unit[1].

To support this idea a stochastic model has been developed to explain the nanoscale observation of paired nanofibrils as a result of diffusion-controlled aggregation and myosin-driven transport of fibronectin-integrin complexes. The evolving patterns of fibronectin clusters and fibrils can be summarized in a morphological diagram as a function of fibronectin-substrate and fibronectin-fibronectin interaction energies.

The restriction of integrin transport to the 2d cell membrane, the coupling of integrin-fibronectin complexes to actin stress fibres, and a shielding effect during fibronectin fibril assembly are proposed as main reasons for the paired nanofibril formation. The spacing between the nanofibrils of one fibril pair was found to be controlled by the size of the focal adhesion when fibril growth starts. Besides paired nanofibrils, further fibronectin patterns were observed, depending on the fibronectin-substrate and fibronectin-fibronectin interaction strengths. A corresponding morphological diagram

shows growth of branched fibronectin clusters and tear-off of fibrils as limiting cases.

Conclusions

In the light of different patterns found in the simulation the experimental observation of formation of elongated nanofibrils – mechanically favoured for an effective load transfer between the cytoskeleton and the extracellular matrix – could be interpreted as a result of optimising related transport mechanisms for fibronectin-integrin complexes in evolution.

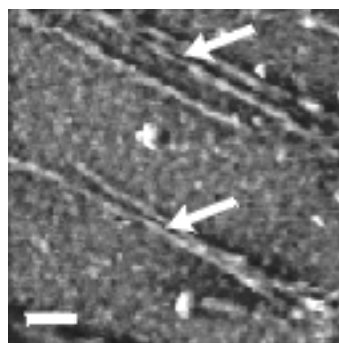


Figure 1: SFM phase image of paired fibronectin nanofibrils (arrows). Scale bar: 500 nm.

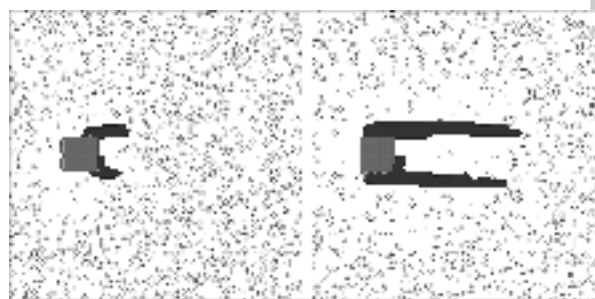


Figure 2: Two snapshots of pattern evolution during a Monte Carlo simulation. The different species of complexes are indicated by different grey levels (white – free, light grey – bound to cluster, dark grey – permanently fixed, black – with stretched fibronectin). The dark grey cluster should resemble a focal adhesion with a simulated width of 350 nm as proposed from the SFM investigations.

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ACTIVATION AND CLUSTERING OF ADHESIVE RECEPTORS BY SURFACE LIGAND DENSITIES ON NANOTEMPLATES TO CONTROL ADHESION AND APOPTOSIS

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Introduction

Many types of cells are anchorage dependent, thus a loss of cell attachment to ECM or adhesion to an inappropriate type of ECM causes apoptosis, or programmed cell death. This subtype of apoptosis is known as anoikis [1]. Anoikis is an essential mechanism for maintaining the correct position of cells within the tissue [2]. Cell spreading and adhesion play a determinant role in switching between cell life and death, by regulating proliferative and survival signals.

Project

The aim of this project is to investigate the effect of the spatial distribution (nanometer range) of specific adhesive ligands, IKVAV, RGD and NCAM-L1, on adhesion and apoptosis. The clustering behaviour of adhesive receptors, such as integrins, modulated by these adhesive ligands on nanotemplates allows studies on the role of cell attachment in the regulation of apoptosis.

Methods

PC12-251 and PC12-27, primary rat embryonic hippocampal neurons and REF52 WT cells were plated on surfaces with a highly ordered quasi hexagonally nanopattern of 6 nm sized gold nanoparticles separated by 58 nm. The space in between the gold nanoparticles was passivated by a polyethylene glycol silane (PEG 2000 derivative), in order to prevent non-specific protein adsorption. Either the IKVAV peptide was covalently linked via thiol group of the n-terminal cysteine residue or cyclic RGD was covalently linked via thiol group to the gold nanoparticles. In the case of the neuronal adhesion molecule NCAM-L1 binding was mediated via his-tag to a monothiol-NTA, which was covalently linked to gold nanoparticles. Specific integrins bind either the IKVAV sequence (isoleucine-lysine-valine-alanine-valine) or the RGD sequence (arginine-glycine-aspartate). The NCAM-L1, a plasma membrane receptor, promotes the adhesion of cells by homophilic binding.

Results

So far different systems have been tested regarding their adhesion promoting ability for PC12-251 and PC12-27 cells, for primary rat embryonic hippocampal neurons and also for REF52 WT cells. The two well characterized systems as the RGD peptide and the NCAM-L1 protein could not promote sufficient adhesion for the neuronal cell lines. Therefore, the IKVAV peptide has been established and tested regarding adhesion for all cell lines. First results show differences regarding the projected cell area and cell cluster formation of different cell lines on IKVAV-, RGD- and on NCAM-L1 functionalized 58 nm patterned surfaces as well as on a IKVAV-, RGD- and on NCAM-L1 functionalized homogeneous gold film.

PC12-251/27 cells and primary rat embryonic hippocampal cells show a significant higher projected cell area and less cell cluster formation on IKVAV patterned surfaces compared to RGD or to NCAM-L1 patterned surfaces. REF52 WT cells adhere well on RGD patterned surfaces but they adhere poorly on IKVAV patterned surfaces.

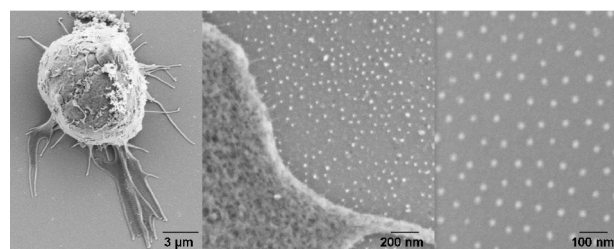


Figure 1: SEM: PC12-251 cell on IKVAV-peptide functionalized nanotemplate.

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EFFECT OF IMPLANT SURFACES ON CELL PHENOTYPES: NEW INSIGHTS IN THE MECHANISMS OF *IN-STENT* RESTENOSIS

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Introduction

In the last decade, the recurrence of coronary artery restenosis following angioplasty has been reduced to 20% by the implantation of expandable metal stents. However, in diabetic patients the incidence of restenosis still accounts for 60% of the cases [1]. The sequence of events leading to the formation of *in-stent* restenosis (ISR) has not been yet clarified, but evidences seem to link it to the degree of tissue damage produced by the stent expansion during the deployment procedure and to the host response to the foreign material [2]. Indeed, histological studies show that ISR is a unique process where activated monocytes/macrophages (MM) induce smooth muscle cells (SMC) to deposit a hyaluronan-rich neointimal tissue [2]. The present study provides insights about ISR focussing on the effect produced by the stent material surface on MM and SMC phenotypes. Different scenarios have been hypothesised and tested in a clinically-reflective *in vitro* model mimicking the ISR process in both control and diabetic subjects. The experiments mimicked MM and SMC adhesion onto: (i) the extracellular matrix (ECM) of the vessel, (ii) the thrombus present around the stent in the early phases of implantation, and (iii) the stent surface.

Materials and Methods

Substrate preparation. ECM (Sigma, UK), fibrin glue (Tissell, Baxter, USA) and electropolished stainless steel (EST, Goodfellow, UK) coupons were used to mimic the different surfaces exposed to cells after angioplasty. All experiments were performed on surfaces pre-conditioned with human plasma and in comparison to tissue culture plates (TCP). **Inflammatory phase model.** MM from control (C) and diabetes Type 1 (T1) subjects were isolated from human peripheral blood and seeded (10^6 cells/mL) on the substrates. Adhering cells were marked with CD68, galectin-1 (Gal-1), and α -actin (α -act) antibodies and analysed by epi-fluorescence microscopy (FM). Cell culture supernatants were tested by ELISA for IL-1, TNF, TGF β 1, and PDGF-BB. C MM activity was also evaluated at physiological (5.5 mM) and hyperglycemic (11 and 20 mM) glucose concentrations. **Tissue regeneration phase model.** Cell line human SMC (3×10^3 cells/mL) were seeded onto the different substrates for 48 h and stained with phalloidin-rhodamine or tagged with PDGF-BB receptor antibodies and examined by FM. Alternatively, SMC were cultured for extra 72 h with or without a PDGF-BB stimulus (2 ng/mL), stained by Hoerscht Propidium Iodide and

counted to assess proliferation rate. *Statistical analysis.* Data were expressed as mean $\pm$ SD from $n=6$ and statistically analysed by ANOVA or paired t test.

Results and Discussion

Adhering cells were identified as MM (CD68-positive) with an anti-inflammatory phenotype (Gal-1-positive). Only EST induced the expression of α -act, suggesting that MM acquire the potential to differentiate into SMC when contacting the stent surface [1]. MM showed low secretion of IL-1 and TNF, whereas TGF β 1 significantly increased on EST (ECM: 265.3 ± 30 pg/ml, Fib: 266.9 ± 47.3 , EST: 364.5 ± 91.4). Only a non significant increase of PDGF-BB levels was found. The study of the effect of TCP and EST on T1 MM showed an increased TGF β 1 secretion in both C (50%) and T1 (61%) cells and no significant difference between the two donors' groups. Conversely, EST stimulated PDGF-BB secretion (687.1 ± 196.2) in C MM which was undetectable on TCP, while higher levels were found in the case of T1 MM regardless of the substrate (TCP: 898.7 ± 253.5 pg/ml, EST 1183 ± 265.3 pg/ml). When incubated in hyperglycaemic conditions, C MM were also activated on both surfaces. These data suggest that in diabetes the material effects on MM is accompanied by patient's predisposing factors. Similarly, EST induced phenotypical changes in SMC; their spindle-like morphology was lost, PDGF receptors were more expressed, and cells increased proliferation under the growth factor stimulus (TCP: 13%, EST: 37%).

Conclusions

The present *in vitro* study shows that EST may induce phenotypical changes in cells relevant to ISR and that diabetic conditions predispose MM to activation. Control of stent surface properties and of clinical parameters (e.g. hyperglycemia) may reduce the risk of ISR without the need of relatively expensive drug-eluting devices.

Acknowledgements

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DESIGN OF BIOINTERFACES: FROM TWO-DIMENSIONAL SURFACES TO THREE-DIMENSIONAL SUBSTRATES

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Introduction

Surface modifications based on biochemical or biological principles are important tools for the fabrication of biosensor chips, biomedical devices such as implants, and of drug delivery carriers. Moreover, well-designed model biointerfaces have substantially contributed in the last decade to a better insight into fundamental aspects of cell-surface interaction.

An overview will be given on tools enabling the surface engineer to tailor the interface of biomaterials, with special emphasis on the approach of eliminating non-specific adsorption (rendering surfaces “non-fouling”) and adding to such a silent surface biological functionalities such peptides, proteins/antibodies, growth factors or vesicles. Preservation of active conformation and optimum presentation (orientation, density) of surface-immobilized moieties are particular challenges in this field.

Molecular Assembly Techniques

Spontaneous molecular assembly and organization of functional molecules is an attractive approach due to its simplicity, cost-effectiveness and compatibility with three-dimensional surfaces and devices. Silanes on oxide surfaces and alkanethiols on gold have been widely studied and successfully used for producing chemically well-defined (model) biointerfaces. Molecular assembly systems are presented in view of their simplicity of application and feasibility to control independently surface topography and chemistry, both of which are known to be important aspects for the interaction of biological moieties, cells and bacteria with surfaces of synthetic and natural materials.

Biochemical Surface Patterns

Creation of two-dimensional (2D) patterns of biointeractive surface chemistry (e.g., covered by extracellular matrix/proteins) in a non-interactive (e.g., PEGylated) background has advanced our understanding of the factors that influence cell behavior of surface *in vitro*. It is, for example, well established from 2D studies that constraining cell shape or degree of spreading via micropatterned adhesive islands determines whether cells proliferate or apoptose [1], whether human mesenchymal stem cells differentiate into adipocytes or osteoblasts [2], and might drive cell polarity during mitosis through orientation of the cytoskeleton [3]. Patterning techniques such as microcontact printing, templated self-assembly, photolithography (Figure 1)

and photochemical immobilization are well established today and will be briefly reviewed. Schemes for the immobilization of bioligands to patterns cover covalent coupling, biospecific interactions (biotin/avidin), metal-organic complexation (NTA-Ni-histag) and others. Bioligands include DNA/RNA, peptides, proteins and saccharides as well as functionalized vesicles and substrate-supported phospholipid or polymeric bilayers.

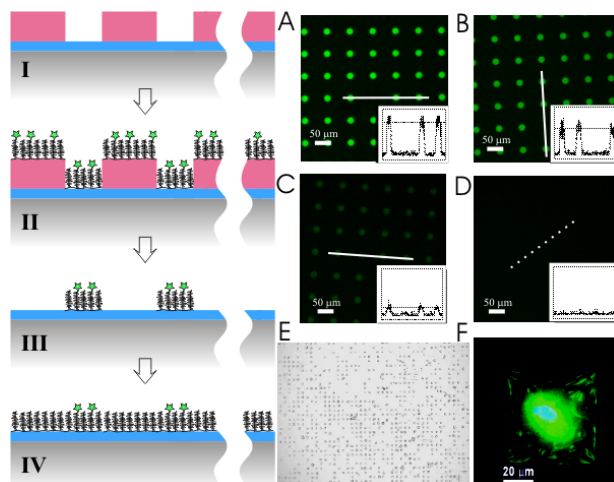


Figure 1:

The Molecular Assembly by Lift-off (MAPL) process converts a metal-oxide/photoresist pattern into a surface with well controlled biointeractive patches in a non-interactive background (I – IV). Inserts A, B, C and D are surfaces with decreasing biotin surface densities revealed by decreased streptavidin fluorescence intensity. E is an array of single cells (MG63 cell line) on round islands of 20 μm diameter. F shows a single fibroblastic cell constrained on a 60x60 μm² square pattern. [9]

2D Patterns versus 3D Microfabricated Substrates

Most of what is known about cells and their functional regulation has been derived from cell cultures performed on flat, mostly rigid culture surfaces, such as the ubiquitous dish introduced by Julius Petri in 1877. However, results obtained in two-dimensions (2D) often lacked the power to predict, for example, the toxicity of drugs in a whole organism or the biocompatibility of synthetic materials. Increasing evidence suggests that placing a cell on a flat 2D substrate versus into a three-dimensional (3D) matrix can have major effect on cell behavior, from adhesion and differentiation to apoptosis.

Materials derived from animals or cell cultures or synthetic materials accessible to cells and tissue in 3D clearly mimic the *in vivo* situation more closely through presentation of copious amounts of molecularly distinct binding sites in a spatially organized fibrillar structure, and our understanding of cell behavior in 3D substrates has greatly benefited from these matrices. However, the microstructural, biochemical, and mechanical properties of those matrices are highly complex and correspondingly difficult to control in a systematic and quantitative manner (Figure 2).

To allow for a quantitative control of the microenvironment and shape of either individual cells or cell clusters in 3D, microfabricated wells are needed that allow tight regulation of relevant physical and biochemical parameters. Substrate rigidity must also be tightly tunable in an attempt to more closely match the microenvironment of cells *in vivo* [4]. Typical soft tissues *in vivo* present a range of elastic properties, with a Young's modulus in the range of hundreds of Pascals (Pa), while modified extracellular matrix production or components contribute to stiffnesses of up to a few thousands Pa in contractile healing wounds. Polyacrylamide gels and poly(dimethyl siloxane) (PDMS) with variable mechanical properties have been used to demonstrate that substrate stiffness regulates cell spreading, cell migration speed, focal adhesion formation, and differentiation of cells, and these findings ultimately led to the current paradigm that numerous mechanoresponsive cell signaling pathways exist [5,6].

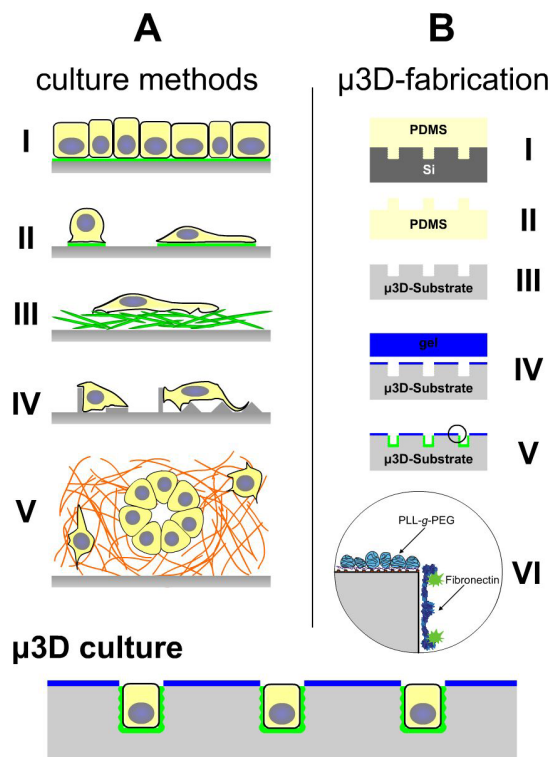


Figure 2:

A: Different methods to culture cells *in vitro*. I) Dense monolayer culture of epithelial cells with 3D aspects of adhesion due to cell-cell contacts. II) Cells with different shapes on 2D adhesive islands of different size. III) Cells on top of so-called 3D cell-derived fibronectin (FN) matrices. IV)

Cells interacting with topographically structured substrates. V) Single cells or aggregates inside a 3D collagen gel.

B: Fabrication of μ 3D culture substrates. I-III) Overview of process steps for the replication of a primary master structure into various materials such as PS, PDMS or PEG hydrogels using an intermediate PDMS master replicated from microfabricated Si.

IV-VI: Scheme of the inverted microcontact printing method. The plateau surface is contacted with a flat stamp to transfer a PEG-graft-copolymer (PLL-g-PEG) rendering those areas non-interactive. The surface of the microwells is backfilled with a cell-adhesive protein, such as FN. [7,8]

Microfabricated Surfaces for Single Cell Arrays

A novel approach is based on fabrication of micro-3D (" μ 3D") culture systems exhibiting arrays of microwells with different shapes and dimensions made from different materials such as polystyrene (PS, 3 MPa), PDMS with tuneable mechanical properties (1–1000 kPa), and poly(ethylene glycol) (PEG) hydrogels (100–1000 Pa). By combining replication techniques with inverted microcontact printing of a protein-resistant PEG-graft-copolymer on PDMS or PS substrates, we have successfully limited protein adsorption and cell adhesion to the inside of the microwells (Figure 2B) [7]. The surfaces, walls and floor, of this first generation of microwells were homogenously coated by fibronectin. First studies of single endothelial cells captured in individual microwells indicate unique distributions of cytoskeletal and other subcellular components, which were highly influenced by the 3D shape of the microwells [8]. Apart from addressing fundamental questions regarding cell behavior in controlled microenvironments, arrays of engineered 3D cell substrates may have significant potential to probe in high-throughput screens the relationship between drug efficacy and the physical and biochemical parameters of given cell environments, thereby improving their predictive power.

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MODULATING EXTRACELLULAR MATRIX AT INTERFACES OF POLYMERIC MATERIALS

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Introduction

As extracellular matrices (ECM) closely interact with cells in living tissues and, through this, influence essentially any aspect of life *engineering of ECM* currently receives a lot of attention in the advent of regenerative therapies. Artificial matrices based on biopolymers isolated from nature were successfully utilized to prepare various types of cell scaffolds to enhance the integration and performance of engineered tissues. Beyond that, translation of progress in matrix and cell biology into new concepts of materials science permits to further refine the functional characteristics of such reconstituted matrices to direct tissue regeneration processes. Our contribution emphasizes research to modulate the functionality of ECM biopolymers through their combination with synthetic polymeric materials. Two examples referring to own studies concern (i) the fate control of endothelial cells by adjusting the availability of surface bound fibronectin for cell-driven reorganization and (ii) the imitation of the bone marrow niche with respect to the cultural amplification of hematopoietic progenitor cells using collagen I based assemblies.

Fate control of endothelial cells through the interfacial mode of fibronectin

The impact of a varied protein anchorage strength on fibronectin fibrillogenesis by endothelial cell was investigated by fluorescence and scanning force microscopy [1] revealing a clear dependence of focal adhesion and fibronectin fibril formation. While covalent fibronectin attachment resulted in an impaired development of focal adhesions and fibronectin fibrils, an enhanced fibrillogenesis was observed on more hydrophilic substrates with physisorptive protein anchorage. More refined experiments [2] allowed for a quantification of the fibronectin fibril pattern together with the focal adhesion density and a correlation of these features to the variation in fibronectin substrate anchorage. Endothelial cells were found to reorganize fibronectin to a much greater extent on hydrophilic substrates. Furthermore, the mean distance between the fibronectin fibrils was found to be smaller on those substrates. Together with the analysis on other copolymer substrates the fibril spacing could be directly correlated to the fibronectin anchorage strength. From

these findings one can conclude that gradated physico-chemical characteristics of polymer substrates can be used to control the anchorage strength of extracellular matrix proteins. By force sensitive cell surface receptors and the intracellular signalling mechanism the modified matrix characteristics subsequently affect not only cell adhesion and matrix reorganisation, but furthermore cellular fate in terms of differentiation.

Imitation of the bone marrow niche using collagen I based assemblies

Fibrillar collagen was reconstituted from mixtures of monomeric tropocollagen and heparin or hyaluronic acid, respectively. The fibrillar assemblies were covalently attached to solid supports. Quantification of surface-bound collagen was accomplished by ellipsometry and HPLC-based amino acid analysis indicating that less collagen was immobilized in the presence of the glycosaminoglycans. SEM and AFM revealed various sizes and shapes of the immobilized fibrillar assemblies if collagen fibrils were prepared in the presence of heparin or hyaluronic acid. Human hematopoietic stem cells (HSCs) were cultivated on the surface-bound collagen fibrils and the migration of adherent cells was studied by time-lapse microscopy. Migration rates on fibrillar structures were significantly lower than on tropocollagen indicating a more intimate contact of HSCs to the fibrillar substrates [3]. Gene expression analysis with microarray chip technology revealed the upregulation of more than 50 genes in presence of collagen I fibrils. Among them, genes for several growth factors, cytokines and chemokines (e.g. interleukin 8, MIP1-alpha) could be confirmed by quantitative PCR [4].

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BIOMIMETIC MATERIALS FOR INJECTABLE TISSUE ENGINEERING: STUDIES OF ACUTE, LASTING AND UNEXPECTED ANGIOGENESIS RESPONSE

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We have focused on the development of injectable tissue repair matrices that enable the regeneration or *de novo* formation of tissue inside the patient's body, a strategy known as *in situ* tissue engineering. For this purpose, we engineered the biological matrix fibrin, as well as cell-instructive polymers based on poly (ethylene glycol) and bioactive peptides that recapitulate the characteristics of collagen in a synthetic scheme for biomimetic, slow release of growth morphogenetic factors. Here I will discuss such strategy for tissue reperfusion therapy and the performance of these materials for stimulating natural blood vessel formation and spontaneous endothelialization of synthetic vessel prostheses.

Targeted delivery of pro-angiogenic proteins such as vascular endothelial growth factor (VEGF) from biopolymeric matrices has presented an inviting strategy to initiate new vessel growth in ischemic or chronic non-healing tissue. However, therapeutic manipulation of adult angiogenesis has remained difficult: (1) Exogenously induced angiogenesis in the adult is a not-selflimiting process that demands tight regulation to prevent pathological vessel growth. (2) Newly formed vessels tend to be leaky. (3) Without sustained supply of VEGF, the newly formed vessel regress. Issues for beneficial intervention with VEGF are: How can we translate supra-normal levels of exogenous factor into local, physiological, tiny dose? And how can we sustain this dose? Our material schemes permit to couple those critical decisions on dosage, location, and duration of released factor to cellular proteolytic activity at the

treatment site, i.e. a biological regulation of release.

We have termed this concept 'cell-demanded release' and we have validated it in great biological detail. The superior performance of such schemes of cell-demanded release of VEGF over conventional formulations is demonstrated in animal models of acute angiogenesis using in video microscopy, morphometry, casting techniques and scanning electron microscopy. Further, we have used biophotonic measurements in transgenic *Vegfr2-luc* (Xenogen) to correlate transcriptional activation of angiogenesis with immunohistochemical analysis of lasting vessel growth at and around the VEGF-fibrin release matrix implant. Together, our data show that with appropriate dose and treatment regimen, single growth factor therapy with VEGF is safe and capable of inducing stable, functional vessel growth.

Surface thrombogenicity and intimal hyperplasia are major reasons for failure of synthetic small diameter prosthetic grafts. VEGF is a potent stimulus for endothelial cell growth, and its provision in a fibrin matrix coating at the luminal graft surface may hold a key to spontaneous graft endothelialization and inhibition of hyperplasia. We have studied engineered VEGF-fibrin matrices applied to the flow surface of synthetic ePTFE grafts in pig trials of spontaneous graft endothelialization. We report an unexpected, untoward effect of VEGF for increasing rather than inhibiting intimal thickening, indicative of a yet unrecognized effect of VEGF for smooth muscle cell activation.

Posters Sessions

IN VITRO GROWTH OF HYALINE-LIKE CARTILAGE ON THE MITOGENIC SURFACE OF HYDROXYAPATITE USING A NEW SWITCH TECHNIQUE

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Introduction

In vitro formation of osteochondral implants for treatment of joint surface defects has to face a major problem: The use of mitogenic carrier materials such as hydroxyapatite is known to support adhesion as well as proliferation of chondrocytes. At the same time, however, production of extracellular components of hyaline cartilage is prevented unless the mitogenic surface effects are masked for instance by coating with chondrogenic biomolecules which is conflicting with effective adhesion of the newly formed tissue to the carrier surface. Here we present a new simple technique allowing production of articular cartilage specific extracellular matrix without losing the strong adhesion effects of the mitogenic carrier surface.

Materials

Chondrocytes were isolated from joint cartilage of adult mini-pigs by subsequent treatment with hyaluronidase and collagenase. In vitro expansion was performed by 3 passages using conventional monolayer culture techniques with growth medium (DMEM) containing 10% fetal calf serum, bFGF (10ng/ml), EGF (1ng/ml). The carriers were made of cured bone cement (Calcibon®, Merck, Darmstadt) with a diameter of 4,55 mm. Its surface was tailored with grains of the same material with an average size of 200µm. Chondrogenic medium consisted of DMEM with 10% fetal calf serum, TGF-β1 (10ng/ml), IGF-I (100ng/ml), ascorbic acid (0.28 mMol/L), cysteine (1 mMol/L).

Results and Discussion

Porcine chondrocytes from the 3rd passage were seeded onto the surface of bone cement carriers (0.5 and 1 x 10⁶ /carrier) and cultured for another 10 days in the presence of mitogenic growth factors (bFGF/EGF) with medium change every other day. During this first phase cells proliferated to a solid multicellular layer still exhibiting a strong adhesion to the carrier. Only at this time the proliferative medium was replaced by the chondrogenic medium containing TGF-β1/IGF-I. The cartilage-carrier-constructs were further cultured for another 20 days also with medium change every other day. Samples from different time points were taken for quantitative analysis of DNA and glycosaminoglycan (GAG) content respectively as well as qualitative analysis of collagen type and histological sections.

Determination of DNA content in 10-day intervals showed a total increase in cell number by a factor of 4 to 5 during the 30 days in culture with the major contribution coming from the early chondrogenic phase right after switching to TGF-β1/IGF-I containing medium. GAG content increased from zero in the mitogenic phase to 350 to 450 µg per carrier during the 20 days culture in the presence of TGF-β1/IGF-I with slight variations depending on the initial seeding density. Semiquantitative analysis of collagen types I and II as obtained by immuno blotting revealed a high preponderance of type II over type I after 10 and 20 days treatment with TGF-β1/IGF-I of the cell layers. HE-staining of histological sections from samples taken at different time points allowed indirect visualization of increasing deposition of extracellular matrix by detection of growing distances between cells during the chondrogenic phase. The development of strong adhesion forces between tissue and carrier was evaluated by a simple peel-off test. As a matter of fact the strength needed for peeling off the cartilage tissue from the carrier with the help of a spatula was found to be significantly dependent on the 10 days treatment of cell-carrier-constructs with mitogenic factors prior to switch to chondrogenic conditions. In order to explain this phenomenon we assume that by switching from mitogenic to chondrogenic conditions a stable gradient is built up between chondrogenic signals prevailing in the superficial zone and mitogenic signals provided by contact with the carrier surface.

Conclusions

The two-step protocol presented here was invented to allow growing cartilage on a hydroxyapatite carrier in order to build artificial osteochondral grafts. By a simple switch from mitogenic to chondrogenic culture conditions the strong adhesion to the hydroxyapatite surface of proliferating fibroblastoid chondrocytes can apparently be preserved after the onset of hyaline cartilage formation.

Acknowledgements

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RELATION BETWEEN WETABILITY AND ROUGHNESS OF BIOMEDICAL METALLIC SUBSTRATES

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Introduction

It is well known that both the surface chemistry and roughness of implants have a major influence on cell behaviour [1]. Nevertheless, one question remains: what is the relative influence of roughness and surface chemistry on cell response? In order to ask to this question, this study aims to perform a surface analysis of model surfaces of austenitic stainless steel (316L) and titanium alloys (TA6V) polished at different paper grades. Extensive studies of roughness and wetability were performed and a possible link between these parameters were studied thanks to a statistical approach.

Materials and Methods

Rods of biomedical 316L and biomedical TA6V were cut, then polished using 80, 120, 180, 220, 320, 500, 800, 1000, 1200, 2400 and 4000 paper grades under identical pressure (150 N) and time condition (3 minutes).

Roughness evaluation was assessed by a three dimensional tactile profilometer. Using a home-made software, roughness was calculated at different evaluation scales. Wetability was measured using classical goniometric technique. Both one liquid (2 μ L drop of water) and two liquid phase methods (2 μ L drop of water in octane) were tested. With the two liquid phase method, either sessile drop technique or dynamic contact angle (DCA) measurements were performed.

Wetability is mainly influenced by the roughness but also by the chemical composition of the surface. Several cleaning protocols were tested and cleanliness was quantified by contact angle measurement. Contact angle was measured without cleaning, after chemical cleaning (acetone and cyclohexane combined with ultra-sonication) and after chemical cleaning combined with an argon plasma cleaning.

Results and Discussion

As a result, it can be concluded without any doubt that the values of the roughness parameters depend on the observation scale. It is outlined that the abrasion process is very reproducible: whatever the series of samples and whatever the roughness parameter, since the recorded values as a function of the evaluation length are very similar. At small observation scales, whatever the amplitude roughness parameters and the paper grade, the recorded values increase linearly as a function of the evaluation length in a log-log scale representation. This characterizes the fractal concept.

Concerning the wetability, it was demonstrated that the contact angle measurement using the one liquid phase

method isn't the best method to quantify the wetability because of the interaction between evaporation and spreading kinetic of the water drop. As a consequence, only the two liquid phase method gives results in this study. Without cleaning, contact angle values around 140° are observed with the sessile drop technique. The values are reduced to 90-100° with chemical cleaning and to 10-35° with chemical cleaning combined with argon plasma cleaning.

No correlation can be observed between roughness and contact angle values without cleaning or with only chemical cleaning. On the contrary, with plasma cleaning, it is found that contact angle values increase with the amplitude roughness parameters both in sessile drop and in DCA (*figure 1*).

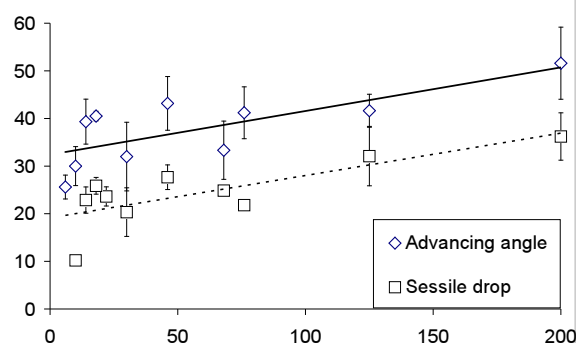


Figure 1: Sessile drop contact angle (lowest values) and advancing angle (highest values) as a function of the size of abrasive grains used for grinding. Contact angles increase as the amplitude roughness parameters increase.

Conclusions

It can be concluded that the wetability and the roughness are linked seeing the contact angle results after cleaning. The main problem is now to find which roughness parameter (amplitude and/or frequency parameter) influences more the surface wetability and at which scale the correlation between roughness and contact angle values is the most significant. This problem is currently under investigation.

In the future, a complementary study will be carried out to find the relationship between those surface roughness and wetability parameters and the osteoblastic cell response to demonstrate what is the relative influence of surface roughness and wetability of metallic substrates on cell response.

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SERUM PROTEINS ADSORPTION AND HUMAN OSTEOBLAST ADHESION ON HYDROXYAPATITE AND β -TRICALCIUM PHOSPHATE SURFACES

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Introduction

Tricalcium phosphate (TCP) has been widely used in association with hydroxyapatite (HA) due to its higher resorption kinetics when compared with HA alone. However, the TCP chemical instability is often the cause of surrounding tissues inflammation, mainly because of the intense reactivity observed at the cell-material interface [1,2]. On the other hand, the cell receptors (integrins) are able to recognize extracellular matrix proteins and proteins adsorbed from body fluids. Probably, a better bone formation promoted by inorganic materials must be linked to their higher efficiency in adsorbing adhesive proteins from the patient's body fluids. Thus, the aim of this work is to evaluate how serum proteins adsorb onto two different bioceramics (HA and β -TCP) and influence the human osteoblasts behavior.

Materials and Methods

Stoichiometric HA and β -tricalcium phosphate powders was compacted by uniaxial pressing and calcined at 1150°C for 4h in order to produce tablets with a dense surface. After calcination, chemical and physical characterization was performed by scanning electron microscopy, X-ray diffraction and Fourier-transform infrared spectroscopy. Half of the β -TCP and HA samples were gold-sputter coated in order to cancel the surface chemical effects. Atomic force microscopy was used to characterize surface roughness (R_a) and real surface area (S_r) of each tablet. For the adsorption experiments, the tablets were coated with culture medium (McCoy's M5A) containing 10% fetal bovine serum (FBS), and incubated from 15 minutes up to 96h at 37°C under a humidified atmosphere of 95% air and 5% CO₂. The adsorbed proteins were desorbed using sodium dodecyl sulfate (SDS) solution, and, subsequently, treated with a total protein assay kit (Micro BCA™, Perbio, France). The absorbances of the systems were measured at 560nm. Human osteoblastic cells (SaOs2) in the 7th passage were seeded on the samples at a density of 4.0_10⁴cells/well. Thermanox® plastic coverslips were used as control. Seeding efficacy was determined by the MTT assay.

Results and Discussion

The average roughness (R_a) measured on the HA (32 $\pm$ 6nm) differed significantly from that for the β -TCP (142 $\pm$ 24nm) surfaces ($p < 0.05$). After coating with gold, no difference ($p = 0.05$) was observed relatively to the original surfaces. Despite the gold coating increase the

initial cellular adhesion, no significant differences ($p > 0.05$) were seen between HA and β -TCP surfaces (Fig.1), suggesting that the surface topography has a

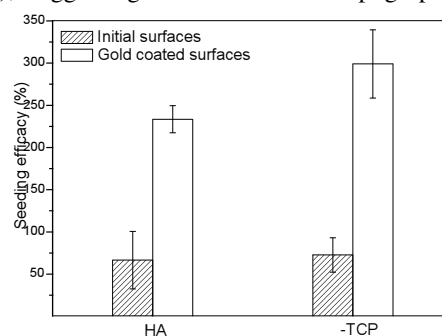


Figure 1: Ratio between the number of adhering cells on the samples and the number of cells adhering on the control after 4h in culture.

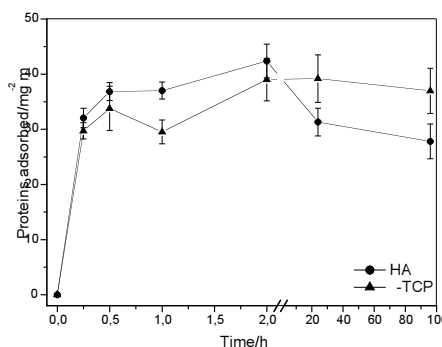


Figure 2: Typical time profile for the adsorption of serum proteins onto HA and β -TCP surfaces.

little influence on cellular initial adhesion after 4 hours. On the other hand, after 2h of immersion in culture medium, the quantity of serum proteins adsorbed onto the HA and β -TCP surfaces was similar (Fig.2). This fact could explain the adhesion behavior if such adsorbed proteins layer also presents similar composition for the two surfaces. To better understand these results, new experiments related to adsorption of albumin and specific extracellular adhesives proteins such as fibronectin and vitronectin are now carried out by means of enzyme-linked immunosorbent assay (ELISA).

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STRUCTURE-FUNCTION RELATIONSHIP BETWEEN FIBRONECTIN ADSORPTION AND CELL BEHAVIOR ONTO MODEL SURFACES

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Introduction

Cells interact with an extracellular matrix or a synthetic support to provide their survival and their correct functions either *in vivo* or *in vitro*. In many cases, cell adhesion to synthetic surfaces is mediated via interactions between cell receptors and adsorbed proteins resulting from physiological fluids or culture media. The amounts of adsorbed proteins, the kinetics of adsorption and the structural changes are the most important features of protein adsorption to surfaces. These parameters affect the molecular organization at the surface and consequently play a central role in the modulation of many cell behaviors.

The aim of our study is to determine onto different model surfaces, the adsorption mechanisms of fibronectin (Fn) - a representative glycoprotein of the extracellular matrix- and the putative consequences onto adhesive and spreading cell behavior regulation of Chinese Hamster Ovarian cells (CHO).

Materials and Methods

Commercial glass cover slips -for cell experiments- and ATR silicon crystals -for FTIR-ATR spectroscopy- were used as silica substrates. Three different model surfaces presenting a rugosity lower than 2 nm were generated:

- A negatively charged and hydrophilic silica surface (noted bare silica; $\theta < 10^\circ$).
- A hydrophobic surface obtained from grafting octadecyltrichlorosilane (noted OTS; $\theta \approx 110^\circ$).
- Another hydrophobic surface obtained by polystyrene (PS) spin coating (noted PS; $\theta \approx 95^\circ$).

Fibronectin (Fn) was purified from human plasma according to a protocol based on fibronectin affinities for heparin and gelatin that yields a large quantity of homogeneous pure Fn (> 98.5% (w/w)).

Protein adsorption was followed during 2 hours in an ATR liquid cell at various bulk concentrations (from 10 to 200 $\mu\text{g/mL}$). The analysis of the amide I' band of the FTIR-ATR spectra enables the determination of both the amount of adsorbed protein and its secondary structures evolutions.

CHO cells behavior -adhesion, spreading and actin cytoskeleton organization- was followed on the different surfaces at three different times with or without coating of Fn. A commercial tissue culture treated support based on a plasma treated PS substrate and coming from Corning was used as a reference surface for cell experiments (noted TCT; $\theta \approx 60^\circ$).

Results and Discussion

We notice that the surface density of adsorbed Fn increases with the Fn concentration and in the course of time. Besides, transport limitation occurs with the lowest Fn concentration. These phenomena occur whatever the surface. For example, Figure 1 presents the results obtained on the PS surface.

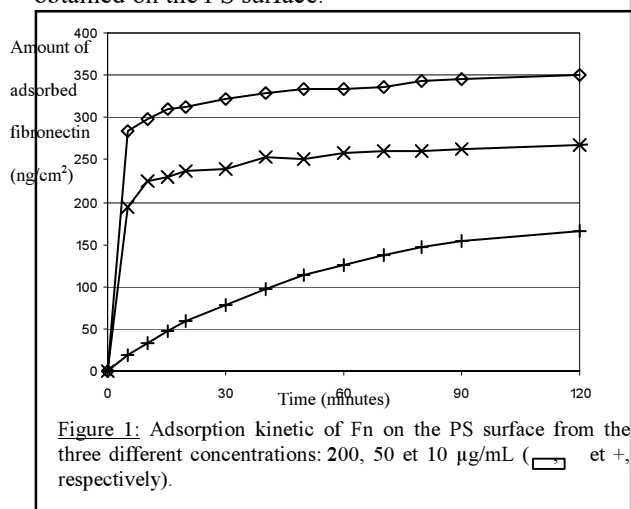


Figure 1: Adsorption kinetic of Fn on the PS surface from the three different concentrations: 200, 50 et 10 $\mu\text{g/mL}$ (□, × et +, respectively).

Concerning Fn conformation, compared to the state in solution, we observe a denaturing effect of the PS and OTS surfaces on the Fn secondary structures during adsorption, whatever the initial Fn concentration. This effect doesn't take place when the Fn adsorption occurs on the hydrophilic bare silica surface. This denaturation is due to the hydrophobic nature of the surface that leads to a conversion of unhydrated β -sheets into hydrated random and β -structures. Moreover on the hydrophobic surfaces the denaturation decreases with the Fn bulk solution.

Concerning CHO cells behavior, adhesion, spreading and actin cytoskeleton organization are favored on surfaces coated with Fn and in the course of time. For a same amount of adsorbed Fn they are favored by the denaturation of adsorbed Fn. The relationship between Fn and cell behavior is studied in different cell culture conditions; that's to say with or without bovine serum albumin (BSA) and/or foetal bovine serum (FBS) proteins.

Acknowledgements

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GRAIN TOPOGRAPHICAL STRUCTURISATION OF TITANIUM OXIDE CERAMIC SURFACE

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Introduction

During the past years, many new applications have been developed for TiO₂, especially in the environmental and biomedical sector. Number of innovative technologies is based on TiO₂, including photoelectrochemical cells, photocatalytic treatment of organic waste, and electrochemical sensors [1].

Different advanced materials can be produced from nanocrystalline TiO₂ precursor powders: thin and thick films, composites, and nanocrystalline ceramics for use in medicine.

It is known that titanium implants have good biocompatibility with living cells. Their corrosion durability is related to spontaneously evolving thin (5-10 nm) oxide layer on metal surface. The passive layer of titanium oxide can be considered as functional ceramics. It is proven that chemical and crystalline composition, as well as thickness of the layer influences response reactions of biological system. This is particularly true for implants in bone, where the surface morphology is of major influence for the osseointegration process.

As most popular surface modification methods one should mention chemical surface modification (hydrogen-plasma-treated and anodised titanium surfaces) and topographically modified surfaces [2, 3].

The goal of this investigation is to obtain structured surface of titanium oxide ceramics for development of biofunctionality of medical implants surface.

Materials and Methods

Titanium oxide ceramic is obtained from commercial TiO₂ powders (Hombitan and Across) with different phases content (anatase, rutile). In order to improve rheological properties, TiO₂ powder is mixed with plasticizer Zusoplast S 13 Spezial and distilled water.

Ceramic samples are made in form of tablets using two-stage (uniaxial and isostatic) pressing technology. Sizes of tablets before firing: diameter 20 mm and thickness 2mm. Ceramic is sintered in electrical furnace at the temperature 1400-1450 °C in air or vacuum. Afterwards, relative density test and surface processing by grinding and polishing of obtained samples is done. Polished ceramic is thermally treated in electrical furnace at the temperature 1350 and 1400 °C varying heating temperature.

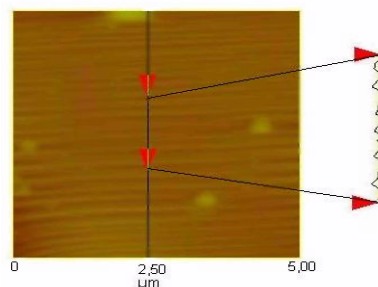
The surface of ceramic samples is investigated by Leica light microscope and atomic force microscope (AFM).

Results and Discussion

Obtained titanium oxide ceramic is characterised by heterogenous colour. Most of the samples are in yellow-grey colour. Ceramic treated in vacuum is in black colour with metallic shine. Relative density of ceramic is in range 96 – 98 %. Linear grain size of melted ceramic is in range 2-20 microns. Creation of so-called twin-grains is

observed on the surface of ceramic. This is related to low-temperature anatase crystalline phase transformation to high-temperature rutile phasis. Moreover, repeated thermal treatment is facilitating changes in colour of ceramic from dark-grey to yellow-grey.

After thermal treatment of polished ceramic samples, the borders between grains on ceramic surface are obvious. Surface topography of separate grains is indifferent in light and dark areas of ceramic. By varying parameters of thermal treatment it is possible to observe changes in dimensions of topographical structure of grains – depth of structural elements from 2 to 20 nanometers, and width – from 100 – 400 nm (Fig. 1).



Surface distance 1.532µm Horiz.distance 1.504µm
Vert.distance 3.811nm Angle 0.145°

Figure 1: Microphotography of thermally treated TiO₂ ceramic surface grain AFM.

Conclusions

The technology for obtaining TiO₂ ceramic and its thermal treatment, which is developed in this study, can be effectively used in targeted structuring of surface of titanium oxide ceramic used in production of medical implants. Thus, it is possible to reach significant improvements of biofunctionality of implants.

It is also possible to develop regular surface topography, create masks using micro- and nano-printing technologies to biofunctionalize surface of medical implants.

Acknowledgements

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INFLUENCE OF IMMOBILIZATION CONDITIONS ON IMMOBILIZATION AND HYBRIDIZATION OF NUCLEIC ACIDS AT TITANIUM BASED IMPLANT MATERIALS

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Introduction

Though titanium and titanium alloys are used as highly biocompatible materials in routine surgery, healing may be problematic for patients with certain risk factors like diabetes or osteoporosis [1]. To mitigate this, a modular immobilization system for bioactive molecules like cell adhesion peptides, growth factors or antibiotics, utilizing the hybridization abilities of nucleic acids (NA), is subject of the presented research [2]. Single stranded NA are to be entrapped partially into an anodically grown titanium oxide layer as anchor strands (AS) and subsequently hybridized with complementary strands conjugated to biologically active molecules. Here interaction of NA with TiAl6Nb7 surfaces was investigated under various conditions. Immobilization using anodic oxidation may cause partial damage of the NA by decreasing pH, mechanical stress, generation of radicals, and formation of complexing titanyl ions. Therefore scavengers and/or complexing agents were added during immobilization.

Materials and Methods

All NA were oligodeoxyribonucleotides (ODN) (Thermo Electron Corp.) partially labelled with ³²P (Hartmann Analytic GmbH) with the properties listed in table 1. CS is a complementary strand to AS and NS is non-complementary to AS, used as negative control.

Table 1: ODN specification

Name	Length	Modification	
		5'	3'
AS	60mer	-PO ₄ ²⁻	-OH
CS	31mer	-OH	-OH
NS	31mer	-OH	-OH

Discs (Ø16 mm x 2 mm) of Ti6Al7Nb (Synthec Inc.) were used for the experiments.

Immobilisation was carried out with 400 nM AS in 40 mM acetate buffer (pH = 4.0) without and with addition of scavenger and/or complexing agent, at a potential of 8 V_{SCE}, using a potentiostat/galvanostat Voltalab 40 (Radiometer Analytical, Copenhagen). For hybridization experiments PBS at pH = 7.4 was used.

ODN surface coverage was measured radioanalytically using a PIPS spectrometer system (Canberra / Ortec).

Results and Discussion

Under all experimental conditions binding of NS was negligible compared to CS (fig. 1), indicating that non-specific binding of the strands occurred only to a minor extent. Without additives or with addition of either scavenger or complexing agent, storage of the samples in PBS at 37 °C after hybridization leads to a fast desorption of CS. Stable binding of CS was achieved only in the presence of both additives during the immobilization step.

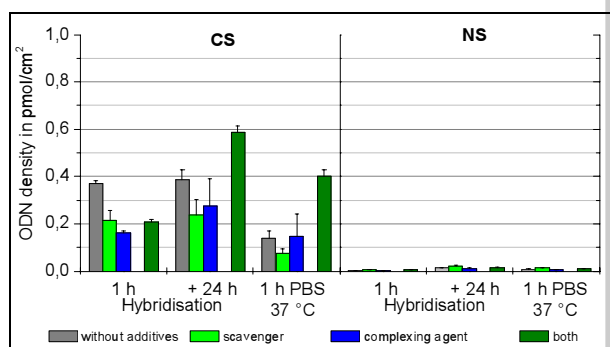


Figure 1: Amount of CS and NS after hybridization and storage in PBS at 37 °C; CS/NS: 400 nM

Conclusion

Fixation of oligonucleotides on titanium based materials using anodic oxidation is a complex process which may lead to degradation of the anchor strands and their occupancy with titanyl ions. Consequently both, scavenger and complexing agent, need to be added during the immobilization step to inhibit the negative effects.

Acknowledgements

Financial support of the DFG (WO494/14 and SCHW638/3-1) is greatly acknowledged. Ti6Al7Nb was kindly provided by Synthec, Inc.

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IN SITU-GELLING HYDROGELS BASED ON POLY(ETHYLENE GLYCOL)

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Introduction

In situ-gelling hydrogels are widely utilized as carrier systems in drug delivery and tissue engineering. They are easy to insert by minimally invasive techniques and can be used to encapsulate cells or biologically active substances. Here we introduce an approach that allows the preparation of hydrogels by covalently crosslinking active esters of poly(ethylene glycol) [PEG] with amine containing compounds under physiological conditions. Such systems can act as a toolbox using different amine compounds and provide a broad variety of biomaterials with distinct biochemical and physicochemical properties. To prove the feasibility of our approach, we first produced hydrogels using branched poly(ethylene imine) [bPEI] and homobifunctional active esters of PEG as model compounds.

Materials and Methods

To obtain homobifunctional α,ω -bis(N-succinimidylsuccinyl-amino)-PEG [PEG2k-NH-SS], a four-step synthesis was established. As previously described, α,ω -bis(amino)-PEG [PEG2k-NH₂] was synthesized from α,ω -bis(hydroxy)-PEG (2 kDa) by Mitsunobu reaction and subsequent hydrazinolysis [1]. To obtain the active ester, PEG2k-NH₂ was treated with succinic anhydride followed by reaction with N-hydroxysuccinimide and N,N'-dicyclohexylcarbodiimide [2]. To confirm the amine reactivity of the active ester, PEG2k-NH-SS was added to α -amino- ω -methoxy-PEG [mPEG2k-NH₂]. The molecular weight of the resulting product was compared to commercially available PEGs (2 kDa, 4 kDa and 6 kDa) by gel permeation chromatography (GPC) using refractive index detection. Gel samples were prepared by combining solutions of PEG2k-NH-SS and bPEI (10 kDa) in PBS at neutral pH, reaching final concentrations of 2 % bPEI and 2 to 4 % PEG2k-NH-SS. After mixing, the samples were characterized by oscillatory rheometry. Solidification was monitored at 20°C by applying a constant torque of 10 μ Nm at an oscillatory frequency of 1 Hz.

Results and Discussion

The synthesized PEG2k-NH-SS was obtained in high yields, as determined by ¹H-NMR spectra (300 MHz, CDCl₃/TMS, data not shown). After reaction of PEG2k-NH-SS with mPEG2k-NH₂, GPC revealed the presence of three fractions with different molecular weights. The main fraction, which is related to molecular weight of 6 kDa, represents the reaction product of PEG2k-NH-SS with two molecules of mPEG2k-NH₂. The two small side fractions, app. 4 kDa and 2 kDa, refer to incompletely reacted PEG active esters and excessive mPEG2k-NH₂, respectively. Due to these results we concluded, that the synthesized product is highly reactive toward primary amines.

By crosslinking bPEI with PEG2k-NH-SS, rapidly gelling, transparent hydrogels could be prepared (Fig. 1).

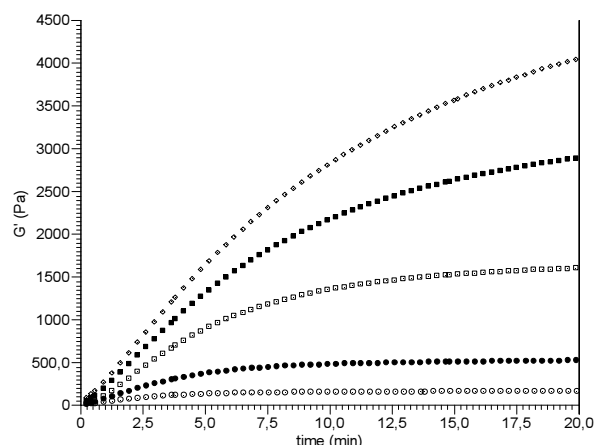


Fig. 1: Gelation of hydrogels consisting of 2 % bPEI and PEG2k-NH-SS (? 2 %, ? 2.5 %, ? 3 %, | 3.5 %, ? 4 %)

Depending on the concentration of PEG2k-NH-SS, the elastic modulus G' steadily increased for different time periods, indicating the progression of crosslinking. In gels of lower concentrations of PEG2k-NH-SS (up to 3 %), G' reached a plateau, whereas in gels of higher crosslinker concentrations the modulus further increased during measurement. The absolute value of G' , which correlates with the stiffness of the produced gels, varies from approximately 160 Pa (2 % PEG2k-NH-SS) to several thousand Pa (4 % PEG2k-NH-SS). The viscous modulus G'' has values up to several orders of magnitude lower than G' ; the loss angle δ is therefore nearby 0°. This indicates an almost ideal elastic behavior of the covalently crosslinked hydrogels.

Conclusion

We successfully established a synthesis of amine reactive PEG derivatives and prepared hydrogels with tunable mechanical properties by crosslinking bPEI with PEG2k-NH-SS. This presents the basis for our further work towards custom-designed biomaterials using a combinatorial approach.

Acknowledgement

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INNOVATIVE INPUT FOR TISSUE ENGINEERING OF BONE AND CARTILAGE FROM THE GROWTH PLATE: THE POSSIBLE ROLE OF ARACHIDONIC ACID METABOLITES

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Introduction

Tissue engineering of cartilage and bone has become an emerging scientific field with important clinical endpoints. In this context the seeding and proliferation of chondrocytes in different types of scaffolds play an important role. The growth plate of long bones represents an integrative structure of chondrocytes in different stages of proliferation and differentiation. The reserve zone plays role as a pool of cells, which enter in cell cycle and showed fast proliferation in the proliferative zone. After some mitoses the cells accumulates glycogen, get hypertrophic and start to synthesize extracellular matrix components before they get apoptotic or were desorbed in the resorption zone. Various paracrine and hormonal parameters are involved in the regulation of this complex structure. Classical, the vitamin D system and insulin-like growth factor but also the effects of glucocorticoids are well documented. From an ontogenic view great efforts have been made in the analysis of the role of hedgehog signalling and the bone morphogenetic proteins.

An alternative aspect in proliferation and differentiation steps of the growth plate is the analysis of arachidonic acid metabolites. In this context prostaglandin E₂ (PGE₂), is known as an important effector molecule in the inflammatory response. Furthermore, its importance in bone and cartilage metabolism has already been demonstrated *in vitro*. Prostaglandins are generated by cyclooxygenases, which exists in two isoforms. Cyclooxygenase-1 (COX-1) is constitutively expressed in nearly all tissues, whereas cyclooxygenase-2 (COX-2) is upregulated in various pathological conditions, but also in ontogenic processes. The effects of PGE₂ are mediated by prostaglandin receptors, which can be detected in cultured reserve zone cells. In the present study we analyzed the effects of different doses of PGE₂ on the proliferation of isolated growth plate chondrocytes. In addition we studied the effects of the unselective COX-Inhibitor indomethacin as well as the COX-1 selective inhibitor SC 560 and the COX-2 selective inhibitor SC236. Furthermore, the effects of EP-1/EP-3 specific agonist sulprostone, the EP2/EP3 receptor agonist misoprostol, the EP2 agonist butaprost, the EP-1 specific agonist ONO-D1-004, the EP-2 specific agonist ONO-AE1-259-01, the EP-3 specific agonist ONO-AE-248 and the EP-4 specific agonist ONO-AE1-329 as well as the EP-1 specific antagonist were analysed. Finally, we examined the the expression and spatial distribution of COX-1 and COX-2 as well as the EP receptors (EP-1 – EP-4) in the growth plate and in cultured growth plate chondrocytes.

Materials and Methods

For proliferation assays chondrocytes were isolated from growth plates of 4 weeks old Sprague Dawley rats

(60-80g), cultured in 96-well-plates and stimulated with 10⁻⁶, 10⁻⁷, 10⁻⁸, 10⁻⁹, 10⁻¹⁰ M PGE₂ and with different unselective and specific COX-Inhibitors (10⁻⁶ M). Furthermore, stimulation with different prostaglandin receptor agonists and antagonists were performed (10⁻⁶ M) for 24 hours. Proliferation was examined by the semiquantitative dsDNA determination and the [³H]-thymidine incorporation. For immunohistological analysis frozen sections of the proximal rat tibia and primary cultures of isolated growth plate chondrocytes were fixed in paraformaldehyde and stained by the alkaline-phosphatase-anti-alkaline-phosphatase method with polyclonal rabbit antibodies against COX-1 and COX-2 as well as EP-1, EP-2, EP-3 and EP-4. As positive controls collagen type I, II and X were analyzed. Gene expression was detected by RT-PCR in homogenized cells of the growth plates and in cultured growth plate chondrocytes.

Results

Stimulation with PGE₂ triggers DNA synthesis in a dose-dependent manner and gave bell-shaped curves with a maximum at 10⁻⁸ M. The proliferation could be suppressed by the COX-unselective antagonist indomethacin and the COX-2 selective antagonist SC236 but not with the COX-1 selective antagonist SC560. The EP-1/EP-3 specific agonist, sulprostone, and the EP-1 selective agonist ONO-D1-004, increased DNA-synthesis, whereas the effect of PGE₂ was suppressed by ONO-8711, an EP-1 selective antagonist. The EP-2/EP-3 agonist butaprost had only little effect on the chondrocyte proliferation whereas pure EP-2 and EP-4 agonists had no effect. The expression of COX-1 and COX-2 as well as EP-1 - EP-4 *in situ* and *in vitro* was demonstrated by both RT-PCR and immunohistology. A markedly different expression pattern of the EP-receptors was observed: Whereas reserve zone showed only weak expression of EP-1 occasionally with negative cells, the EP-2, EP3 and EP-4 receptor were detected homogenously in all zones of the growth plate. Growth plate chondrocytes *in situ* and *in vitro* expressed COX-1 and COX-2.

Conclusion

PGE₂ stimulates the proliferation of growth plate chondrocytes *in vitro* in a dose-dependent fashion via the EP-1 receptor. Furthermore, for proliferation of growth plate chondrocytes COX-2 is from special importance. These findings open new interesting aspects for a possible improvement of proliferation of chondrocytes in scaffolds used in bone and chondrocyte tissue engineering. Further studies should aim at additional functions of PGE₂ via the EP-2, EP-3 and EP-4 receptor.

IN VITRO STUDIES OF MESENCHYMAL STEM CELLS ON THE CALCIUM PHOSPHATE XEROGEL COMPOSITE BONIT MATRIX®

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Introduction

Cell attachment, proliferation and differentiation on biomaterials in vitro are indispensable for further application of cell-material-constructs for bone replacement and regeneration of bone defects. Bonit matrix® is one of the new developed materials on the base of bone-like calcium phosphates which is fully degradable and already tested successfully in vivo [1]. The combination of this material with multipotent precursor cells such as human mesenchymal stem cells (hMSC) is a promising approach for tissue engineering. We investigated the ability of Bonit matrix® surfaces to induce osteoblastic differentiation of hMSCs tested by the expression of osteogenic markers.

Materials and Methods

Bonit matrix® consisting of 87 % calcium phosphates (60 % hydroxyapatite and 40 % β -tricalciumphosphate) and 13 % SiO_2 was prepared as compact discs (d=11 mm) by DOT Ltd. Rostock. Prior to seeding of cells onto the material, the discs were conditioned in standard cell culture medium DMEM with 10 % FCS and 1 % antibiotics/antimycotics (expansion medium = EM) for 24 h with medium exchange done twice.

hMSCs (Fa. Cambrex) were proliferated up to 3 passages in EM. 2×10^4 cells were seeded onto each Bonit matrix® disc and cultivated for 22 d in EM without additional supplements to promote osteoblastic differentiation. Cell adhesion and growth were visualized by scanning electron microscopy (SEM) after 24 h, 3 d, 9 d and 22 d.

The determination of osteogenic differentiation markers was carried out by quantitative real time RT-PCR. Cells were harvested after 3 and 14 d of culture on Bonit matrix® discs. Total RNA extraction and RT-PCR were performed as already described [2].

Results and Discussion

After 24 h on Bonit matrix® discs adhered cells displayed a flat and wide spread phenotype with extensive formation of filipodia. Up to day 9 cell number increased so that the disc surfaces were completely covered by cells and after 22 d they formed a dense layer (Fig. 1). The expression of proteins characteristic for differentiated osteoblasts revealed that the surface of Bonit matrix® induced differentiation of hMSC to osteoblasts when cells were cultured in EM. While alkaline phosphatase (ALP), collagen type 1 (Coll.1) after 3 d and bone sialoproteine (BSP) after 14 d increased slightly on Bonit matrix® in comparison with

tissue culture plastics (Fig. 2), osteocalcin (OCN) expression was distinctly increased after 14 days which suggests a specific effect of the material surface to induce OCN expression.

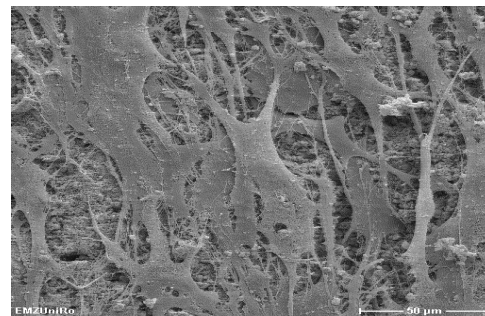


Figure 1: SEM image of the dense cell layer formed by hMSCs after 22 d culture on Bonit matrix®

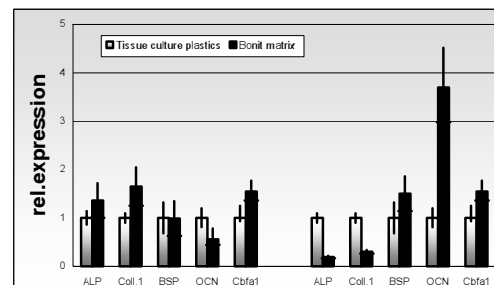


Figure 2: Osteogenic markers expressed by hMSCs after 3 d (left side) and 14 d (right side) on Bonit matrix®

Conclusions

The surface of Bonit matrix® discs promotes the differentiation of hMSC to osteoblasts in expansion medium which suggests that signals induced by cell - extracellular matrix interaction on this material could replace effects of soluble differentiation factors.

Acknowledgements

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BIOCOMPATIBILITY OF CLUSTER-ASSEMBLED NANOSTRUCTURED TiO₂ WITH PRIMARY AND CANCER CELLS

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Introduction

We have characterized the biocompatibility of nanostructured TiO₂ (ns-TiO₂) films produced by the deposition of a supersonic beam of TiO_x clusters. Physical analysis shows that these films possess a nanoscale granularity and porosity mimicking those of typical extracellular matrix structures and adsorption properties that could allow surface functionalization with different macromolecules such as DNA, proteins and peptides. To explore the biocompatibility of this novel nano-structured surface, different cancer and primary cells were analyzed in terms of morphological appearance and growth properties, with the aim to evaluate cluster-assembled TiO_x films as substrates for cell- and tissue-based applications.

Materials

ns-TiO₂ films were grown on round glass coverslips by depositing under high vacuum a supersonic seeded beam of TiO_x clusters produced by a pulsed microplasma cluster source (PMCS) [1, 2]. Immortalized Mouse Embryo Fibroblasts (MEFs), human osteo-sarcoma U2OS, human fibroblasts Tig3-hTert, and primary human melanocytes were cultured and plated on the coverslips, which were previously coated either with ns-TiO₂ or 0.2% of gelatin PBS. Cells were imaged in vivo by a bright field inverted microscope. Immunofluorescence experiments for cyto-skeletal analysis and cell cycle analysis by BrdU (5'Bromo 2'deoxyuridine) incorporation were performed [3].

Results and Discussion

The deposition of TiO_x clusters on the substrate produced low-density and highly porous films with grain diameters of about 20 nm and 4-5 nm rms roughness. Cells plated on this substrate adhered and grew with the same morphology as cells grown on a gelatin-coated coverslip; the same cytoskeletal parameters (tubulin, actin and focal adhesion) were observed for both cells grown on cluster assembled TiO₂ and gelatin. Cell cycle analysis by BrdU incorporation showed that our substrate does not inhibit nor stimulate cell growth.

Conclusions

Cluster assembled TiO₂ is a biocompatible material that allows normal growth of tumor and primary cells. The use of this material as cell culture substrate is of particular interest for the coupling of cultured cells on

microfabricated devices, since supersonic cluster beam deposition is fully compatible with planar micro-fabrication technologies [4, 5].

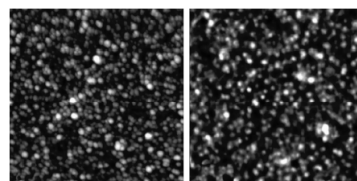


Figure 1: AFM image of a ns-TiO₂ film (left, 500x500 nm², vertical scale 15 nm) and a gelatin coating (right, 500x500 nm², vertical scale 3 nm) on a glass coverslip.

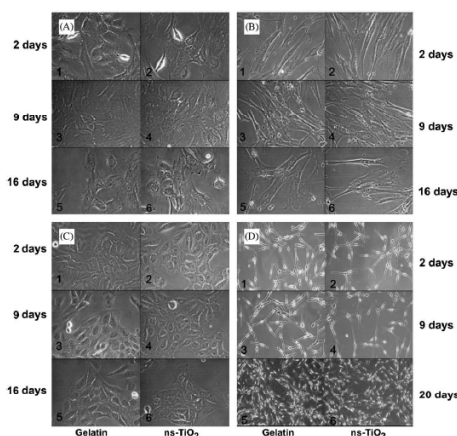


Figure 2: Morphological analysis by bright field microscopy (A) MEFs, (B) Tig3-tert, (C) U2OS (D) human primary melanocytes

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ANTIBACTERIAL CONDITIONING OF PURE HYDROXYAPATITE (HA) WITH DIFFERENT POROSITY

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Introduction

Calcium phosphate compounds, in particular HA and β -TCP, are the principal synthetic materials used for bone substitutes. The frequency of per-operative infections is increasing and bioceramics with anti-bacterial effects are useful. To assess the quality for grafting of further drug delivery systems, a pure, small grain HA was elaborated with specific internal material porosities and then tested on its cytological and microbiological effectiveness.

Material and Methods

Highly pure HA wafers were formed with the slip casting method. The purity was controlled with X-ray diffraction, IR and Raman spectrometry. Micro- and meso-porosities were obtained by varying the sintering temperature (1200-1250°C) and/or adding graphite as porogenous agent (Fig. 1). The biological tests concerned cell viability, proliferation and morphology (SEM) after surface conditioning [1], and the antibacterial efficiency of Vancomycine impregnated unpolished wafers on *S. aureus* (*S.a.*) and *S. epidermidis* (*S.e.*).

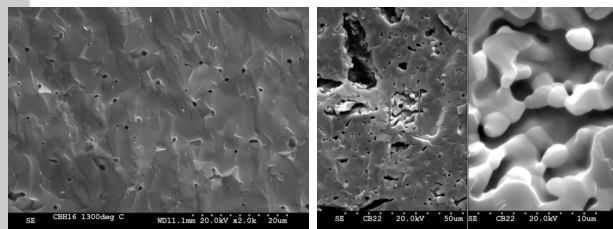


Figure 1: SEM observation of micro-porous (left) and meso-porous (right) Hydroxyapatite.

Results

The cell viability tests using the colony forming method confirmed the excellent cytocompatibility of HA, the graphite powder and the antibiotic (AB) Vancomycine. MC3T3-E1 osteoblasts were grown on HA conditioned with culture medium and FCS for 2h [1]. All HA samples produced a higher proliferation rate than the TCPS controls; the micro-porous HA inducing the highest cell growth near 150%. The AB impregnated HA also stimulated cell proliferation but in lower extend (Fig. 2). No significant differences are observed as to the cell morphology compared to the control.

HA samples were impregnated for 7.5, 15 and 60 min with the AB (10 and 20 mg/L) under normal pressure and under vacuum. After drying, the AB release was studied in human plasma up to 72 h by the disk and liquid agar diffusion tests to assess the antibacterial effectiveness.

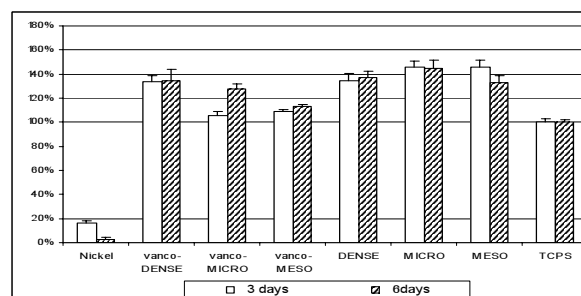


Figure 2: 3- and 6-days proliferation of MC3T3-E1 cells on dense, micro- and meso-porous HA conditioned with FCS and impregnated for 1 h with Vancomycine (n=6).

No differences were found after the AB impregnation under normal pressure or under vacuum. The AB release and the antibacterial efficiency were the highest but not longest for the highest concentration. The impregnation time, however, plays a significant role for the duration of release and the antibacterial efficiency. In all cases, the AB effect was significantly the strongest and longest on the micro-porous HA (Fig. 3).

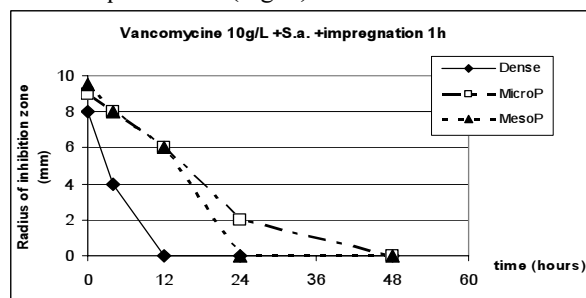


Figure 3: Antibacterial efficiency of dense, micro- and macro-porous HA impregnated with Vancomycine (1h).

Not only the purity but also the micro-porosity have an influence on the cell behaviour and on the adsorption and release time of bioactive molecules such as antibiotics. These efficiencies will now be improved by the grafting of cyclodextrins as controllable drug delivery system in the HA porosity volume to prolong the effect of bioactive molecules [2].

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IN VITRO BEHAVIOUR OF OSTEOBLAST-LIKE CELLS ON PLLA WITH APATITE COATING AND APATITE/COLLAGEN COMPOSITE COATING

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Introduction

As the two primary components of extracellular bone matrix, apatite and collagen exhibits unique advantages. We have combined collagen with the 5 times simulated body fluid (5SBF) and fabricated a novel composite coating containing submicron apatite particulates and collagen fibers on PLLA films and scaffolds [1]. The aim of this study is to evaluate the effect of the two biomimetic coatings, apatite coating and apatite/collagen composite coating, fabricated using the accelerated biomimetic process, on the cellular behaviour.

Materials

PLLA was purchased from PURAC (Netherlands). Type-I Collagen was purchased from Tsinghua University in Beijing, China. The osteoblast-like cells, Saos-2 cells, were supplied by American Type Culture Collection (ATCC, USA).

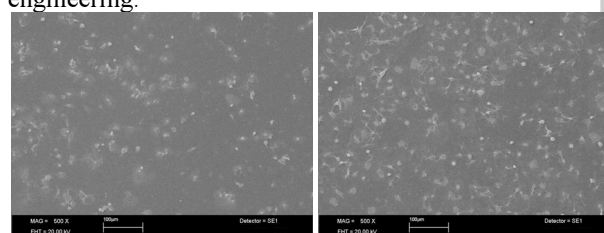
Results and Discussion

It was confirmed from the SEM observations (Figure 1) that after 2 hours of culture more cells were found attached on the composite coating and they had stretched their pseudopodia well, whereas fewer cells were observed on the PLLA substrate and their pseudopodia were less obvious. Cell number and morphology on the apatite coating were somewhat between that on PLLA substrate and composite coating. Alkaline phosphatase (ALP) activity of saos-2 cultured on PLLA films with apatite coating and composite coating after 8 days of culture were significantly higher than that on the PLLA films control with $p < 0.05$ and $p < 0.01$, respectively (Figure 2). Compared to the apatite coating, the ALP activity of saos-2 cultured on PLLA films with composite coating was significantly higher ($p < 0.05$). The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrasodium bromide (MTT) results showed that after 8 days of culture the viability of saos-2 cultured on composite coating was significantly higher than that of control ($p < 0.05$) and apatite coating ($p < 0.05$).

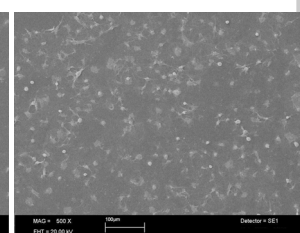
Conclusions

The biomimetic apatite coating and apatite/collagen composite coating fabricated through the accelerated biomimetic processes could improve the interactions between osteoblasts and PLLA. The composite coating was more effective than apatite coating in improving such interactions. PLLA coated with submicron collagen

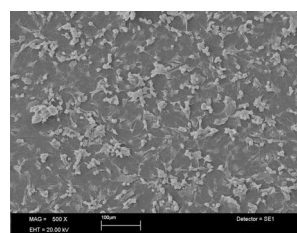
fibrils and submicron apatite particulates is expected to be one of the promising substrates for bone tissue engineering.



(a) Without coating



(b) with apatite coating



(c) With apatite/collagen composite coating

Figure 1: SEM image of Saos-2 cells after 2h culture on PLLA film.

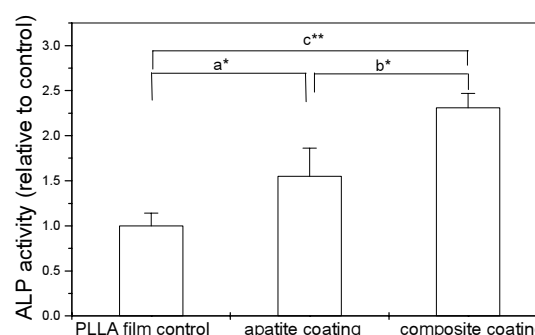


Figure 2: The ALP activity of osteoblast-like cells seeded on PLLA film without and with coating. (* $p < 0.05$; ** $p < 0.01$).

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TOWARDS SELECTIVE NEURONAL ADHESION ON BIOFUNCTIONALIZED NANOPATTERNED HYDROGELS

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Cochlear implants are auditory prostheses that allow coding of acoustic information into electrical stimuli by aid of electrode arrays implanted into the cochlea. Although these implants are very promising, their effectiveness decreases with time due to fibrous encapsulation that isolates the prostheses from the nervous system. Therefore, development of strategies to improve the neuron-implant interface is required. Efforts to improve cell survival and integration have focused on biocompatible scaffolds that support neuronal cell growth and function while inhibiting fibrous growth. These include immobilization of extra cellular matrix proteins as well as specific cell-receptors at surfaces, thus enabling biomaterials to influence cell attachment, differentiation and eventually tissue organization.

The aim of the present study is to develop a biocompatible surface for selective adhesion of neurons to be ultimately used as coating material in cochlear implants. The coating material, ideally,

would preferentially attract neurons to the surface of the implant leading to a better integration of the entire implant in the host tissue.

From nature we know that cells are sensitive to cues from their substratum i.e.: mechanical, chemical and structural cues. Moreover, several studies have demonstrated that neurons display a spreading preference for 'soft' materials. Using a novel technique, nano-patterned gold particles were transferred from a glass surface to a polymeric surface based on poly (ethylene glycol) (PEG) with nano-scale precision. The gold nano-particles were functionalized with cyclic RGDfK peptide, L1 cell adhesion molecule and IKVAV peptide. Cell adhesion and growth of fibroblasts (REF-52 WT) and neurons (PC12-27) on the different substrates was study. We observed that both mechanical and structural properties combined with opportune chemical functionalization influence the cell adhesion behavior.

CALCIUM PHOSPHATE PRECIPITATION ON BIOGENIC CALCITE

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Introduction

The eukaryote phylogeny presents three inorganic biomaterials: calcium carbonate, calcium phosphate and silica. Calcite is the most stable form of calcium carbonate and is biomineralized by various marine organisms¹. Birds use biomineralization processes to produce eggshells with an inorganic part consisting of biogenic calcite. In this study we investigated the calcium phosphate formation on avian eggshell surfaces that were chemically pre-treated with phosphate solutions at different pH.

Materials and Methods

The external side of eggshell samples was partially submerged in 200 mL of 1.7M H₃PO₄ for 2h and 1M (NH₄)₂HPO₄ for 48h, respectively. Surface modifications were characterized by ESEM and FTIR analyses. EDX-mappings of phosphorous were used to quantify layer thicknesses. The *in vitro* bioactivity of chemically treated as well as untreated eggshell samples were tested by using simulated body fluid (SBF) solutions with a composition nearly equal to the inorganic part of human blood plasma². Pieces with a dimension of 1 cm x 1 cm were soaked in 200 ml SBF for 2 weeks under physiological conditions (pH 7.4, T = 37 °C).

Results and Discussion

The outer surface of avian eggshells mainly consists of calcite. Chemical treatment in phosphate containing solutions leads to the formation of calcium phosphates. FTIR spectra of samples treated with H₃PO₄ indicate the formation of dicalcium phosphate dihydrate (DCPD, CaHPO₄·2H₂O) with characteristic phosphate bands at 1133, 997, 890, 580, 560 and 523 cm⁻¹. The DCPD layer precipitated on the eggshell surface had a thickness of 50 µm.

In the case of (NH₄)₂HPO₄ treated samples the FTIR spectra showed phosphate bands at 1100, 1028, 600, 560 cm⁻¹ and carbonate bands at 1419, 1443 and 1535 cm⁻¹, indicating the formation of hydroxy carbonated apatite (HCA, Ca_{10-x}(HPO₄)_{x-y}(CO₃)_y(PO₄)_{6-x}(OH)_{2-x-z}(CO₃)_{z/2}, with 0 < y < x < 1 and z ≤ 2 - x). The thickness of the HCA layer amounts to 10 µm.

The pH value during chemical pretreatment with H₃PO₄ and (NH₄)₂HPO₄ was equal to 1 and 8, respectively. Thus, the obtained results are in good agreement with solubility isotherms of calcium phosphate phases. DCPD was shown to be the most stable calcium phosphate at a pH below 4.5 and apatite above 4.5³. Calcite represents the most stable phase at pH > 9.5.

Exposure to SBF leads to the formation of bone-like apatite on all tested samples. After 1 week soaking in

SBF untreated eggshell samples were completely covered by apatite, whereas the rate of apatite formation was lower for DCPD and HCA coated samples. After two weeks exposure to SBF all samples were covered by a dense apatite layer. The higher bioactivity of untreated eggshell samples might be explained by the higher solubility of calcite at physiological pH compared to DCPD and HCA. Calcium ions leached into the medium might increase the degree of supersaturation with respect to apatite. As a result bone-like apatite precipitates on the sample surface.

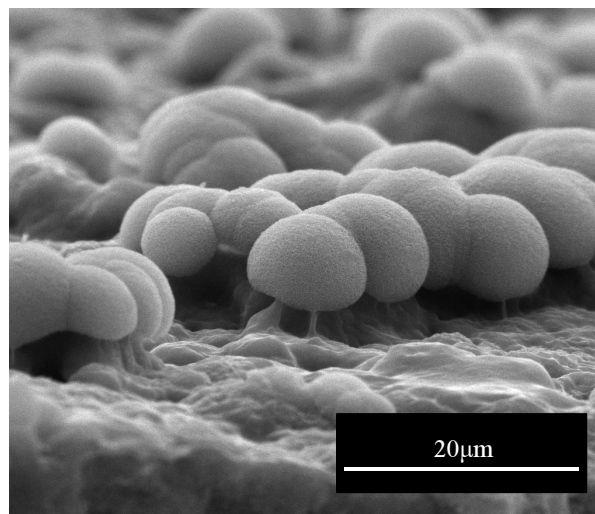


Figure 1: ESEM micrograph of an eggshell treated with H₃PO₄ after 1 week soaking in SBF.

Conclusions

Chemical treatment of biogenic calcite surfaces in H₃PO₄ and (NH₄)₂HPO₄ leads to the formation of DCPD and HCA, respectively. During exposure to SBF all tested samples formed a bone-like apatite layer on their surface, indicating their *in vitro* bioactivity.

Acknowledgements

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QUANTIFICATION OF OSTEOCLAST RESORPTION ON SYNTHETIC HYDROXYAPATITE

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Introduction

The regeneration of bone fractures or large bone defects is a serious problem in orthopaedic and maxillofacial surgery. One goal of a bone substitute material used for bone regeneration is to be resorbed by osteoclast cells and replaced by new bone. Calcium phosphate (CaP) ceramics are one of the most implanted biomaterials in this field. These CaP materials have an excellent biocompatibility, because they are very similar to the anorganic phase of the human bone. However, the degradation (chemical dissolution) and resorption of these synthetic materials are controversially discussed.

For this reason we cultivated murine monocytes on a hydroxyapatite (HA) ceramic surface to generate osteoclast-like cells. After a defined cultivation period the activity of the osteoclast cells was correlated with number, volume and depth of the formed lacunas.

Materials and Methods

The ceramics disks used in this study were prepared from commercially available HA powder. The powder was mixed with organic additives in a solvent and after mixing, drying and sieving, granulation was carried out. The granules were uniaxially pressed and the resulting green bodies were sintered at 1300 °C. The ceramic samples were characterised by e.g. x-ray diffraction (XRD) or scanning electron microscopy (SEM). The monocyte cell line RAW 264.7 was cultured in alpha MEM supplemented with 10 vol% fetal bovine serum and 1 vol% antibiotics. Before cell seeding, the samples were cleaned, sterilized at 134 °C in an autoclave and placed in 24 well cell culture plates. Thermanox[®] plastic cover slips were used as a control. RAW 264.7 cells were cultured on the test surfaces in standard culture media and in the presence of 25 ng/ml M-CSF (macrophage colony stimulating factor) and 40 ng/ml RANKL (receptor activator of NF-kappaB ligand) for 14 days. The attached cells were analysed by counting with a coulter counter after detaching them from the material surface. The gene expression of tartrate-resistant acid phosphatase (TRAP) was analysed by RT-PCR. Actin-irrig structures were labelled by FITC-phalloidin, nuclei were stained by DAPI and observed by fluorescent microscopy. Cell morphology was examined by SEM. In addition, after the cells have been detached from the surface, the lacunas resulting from osteoclast activity were quantified by a 3D image software (Alicona). Depth and volume of these lacunas were calculated from SEM-images of the surface.

Results and Discussion

Analysis of the HA samples by SEM showed an almost dense surface with typical ceramic structure. X-ray diffraction of the material after sintering showed that the ceramic samples consisted of pure phase HA.

When RAW 264.7 monocytes were stimulated with RANKL and M-CSF they formed multinucleated giant cells after 14 days of cultivation. The morphology of an osteoclast-like cell attached on HA was flat and round with many pseudo- and filipodia (fig 1, A). The functional activity of osteoclast-like cells was concluded from the topographical changes in the HA surfaces. The resorption areas on HA are distributed over the whole surface and the lacunas are characterised by a "crystalline structure" on the bottom and proceed along the grain boundaries (fig. 1, B).

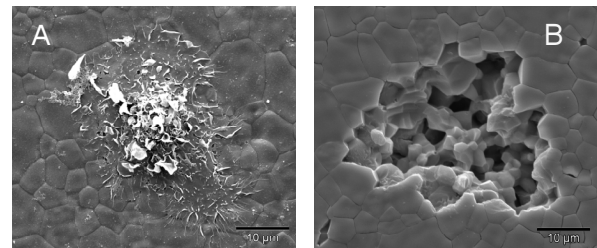


Figure 1: SEM images of A) a RAW 264.7 cell cultured for 14 days and B) a lacuna on a HA surface.

To analyse the *in vitro* resorption, a 3D image software was used. For example, we measured a depth of approximately 10 µm and the diameter ranges from 40 – 60 µm of a presentable lacuna (fig. 2). In this case the resulting volume was calculated approximately 3500 µm³.

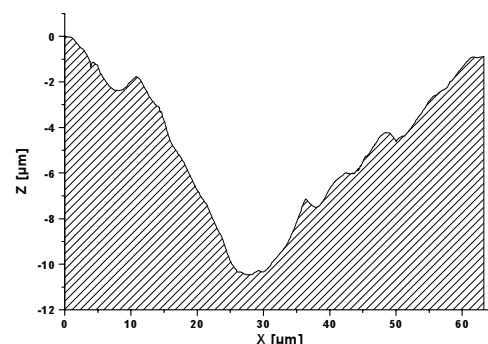


Figure 2: Profile of a lacuna formed from a RAW 264.7 cell cultured for 14 days.

Conclusion

Osteoclast acid secretion into the sealed extracellular substratum causes an ion release from the calcium phosphate surface. To measure this activity of osteoclast cells on bone substitute materials the resulting lacunas can be analysed by our method on the artificial material.

ANALYSIS OF COLLAGEN FIBRIL-BASED EXTRA-CELLULAR MATRICES WITH INCORPORATED GLYCOSAMINOGLYCANS (GAGs) FOR TITANIUM IMPLANTS

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Introduction

The extracellular matrix protein collagen has been used as a coating for titanium implants. During collagen fibril formation in vitro, glycosaminoglycans (GAGs), which are believed to influence osteoblast behaviour and also bind growth factors (GFs), bind to the fibrils. The GAG heparin interacts with the GFs BMP-4 and VEGF, which are involved in osteoblast differentiation and angiogenesis respectively. To compare the suitability of different collagen types and GAGs for use in coatings, we quantified the amount of low and high molecular weight heparin (lo hep, hi hep) and the structurally similar GAG chondroitin sulphate (CS) bound by fibrils of collagen types I, II and III as well as the resulting changes in fibril morphology. Primary human osteoblast (HO) behaviour on titanium coated with CS-containing type I fibrils was also studied.

Materials and Methods

Collagen types I (calf skin), II (bovine tracheal cartilage) and III (human placenta) as well as GAGs were obtained from Sigma-Aldrich Chemie GmbH, Germany. Fibrillogenesis took place at 37°C in a 30 mM phosphate buffer at pH 7.4 at different GAG:collagen ratios. Fibril mass was determined by the Lowry protein assay. GAG content of fibrils was measured colorimetrically by both hexosamine and dimethylmethylene blue assays. Fibril morphology was studied by atomic force microscopy (AFM). Titanium surfaces were coated with fibrils by adsorption and seeded with HOs (PromoCell GmbH, Germany) with subsequent determination of proliferation and collagen synthesis.

Results and Discussion

Collagen type II fibrils bound more GAGs than fibrils of types I and III. Types I and II bound significantly more hi hep than lo hep (Fig. 1) and all three types more hi hep than CS (Fig. 2). Both colorimetric assays yielded similar results. Differences might be explained by differences in molecular weight; longer glycosaminoglycan chains may form more stable bonds with collagen by "bridging" more binding sites on collagen. Furthermore, GAGs have different charge densities; a higher charge density would lead to increased ionic binding. The amounts of GAG immobilised in this manner are comparable to those achieved by EDC/NHS crosslinking [1]. GAGs had no significant effect on fibril morphology of any collagen

type. Type II may bind more GAGs than type I because of its larger relative surface area due to its thinner fibrils. Type II may also have a higher inherent affinity for GAGs than types I and III. HO's cultured on collagen-coated titanium surfaces containing CS exhibited greater proliferation and collagen synthesis compared to those cultured on uncoated titanium.

Conclusions

The ability of collagen fibrils to bind GAGs varies according to GAG and collagen type. GAGs do not influence fibril morphology significantly. Coatings consisting of type I fibrils containing CS appear to modulate primary human osteoblast behaviour. These results may be of importance when designing collagen-based extra-cellular matrices.

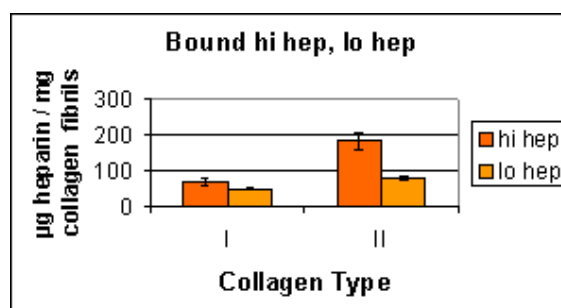


Figure 1: Quantification of lo hep and hi hep bound per mg collagen fibrils of collagen types I and II.

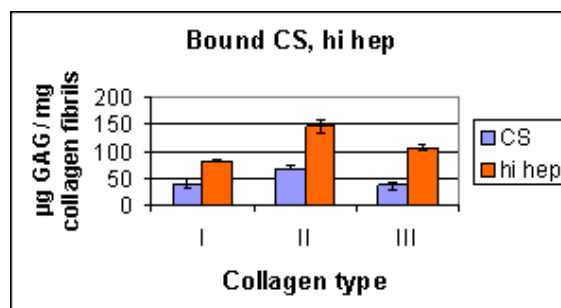


Figure 2: Quantification of CS and hi hep bound per mg collagen fibrils of collagen types I, II and III.

Acknowledgements

BMBF, Germany for funding and T. Hanke for advice.

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SCREENING OF POROUS GELATIN HYDROGELS AS SCAFFOLDS FOR TISSUE ENGINEERING

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Introduction

Gelatins have previously found widespread applications as food ingredient, pharmaceutical capsules, paper additive, cosmetic ingredient or as additive to photographic silver emulsions. The application of gelatin as biomaterial is a more recent development. Despite the large number of gelatin-related research papers, a screening study of a limited number of gelatin hydrogels using a panel of cells has not been performed before.

Materials and Methods

In the present work, two types of porous gelatin hydrogels with varying pore geometry and pore size were compared. Type I hydrogels contain top-bottom transversal channels with a decreasing pore size from top (330 μm) to bottom (20-30 μm)^[1]. Type II hydrogels contain spherical pores with a diameter of 135 μm .

The *in vitro* cell biocompatibility of the scaffolds was evaluated by applying a panel of human cells on the hydrogels: human umbilical vein endothelial cells (HUVEC), osteoblasts (MG-63 and CAL-72), human foreskin fibroblasts, glial cells (U373-MG) and epithelial cells (HELA). Cells were seeded at a density of 160000 cells/scaffold (1.5 cm^3). At different time points, the hydrogels were incubated in a calcein-AM solution (1 $\mu\text{g/ml}$) for 5-10 minutes at 37°C in the dark. Cell visualisation was performed using confocal microscopy.

Results and Discussion

The results of HUVEC seeded on type I hydrogels are shown in fig. 1 indicating that HUVEC attached and spread on the gelatin scaffolds. The cell morphology was similar compared to cells on tissue culture plastic.

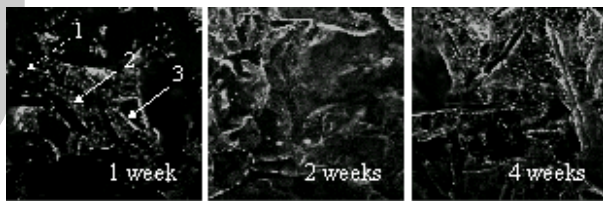


Figure 1: Visualisation (5X) of HUVEC seeded on type I hydrogels.

Three different type of cellular organisation could be distinguished one week after cell seeding: (1) single cells (2) confluent cell layers and (3) cells aligning the pores of the hydrogel. At longer incubation times, no single cells were observed indicating HUVEC proliferation.

The results for the other cell types on type I hydrogels after four weeks, are shown in fig. 2. The data for all cell types on type II scaffolds are shown in fig. 3.

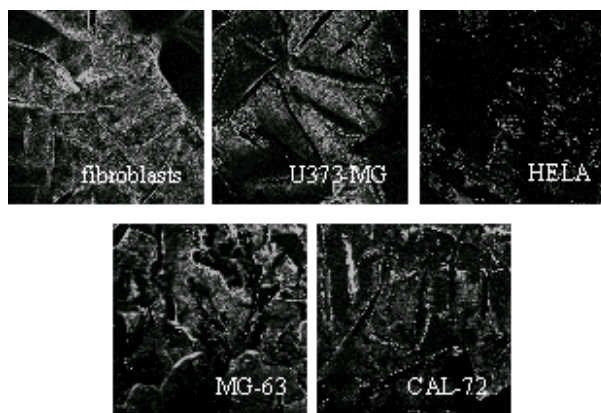


Figure 2: Visualisation (5X) of different cell types, 4 weeks after cell seeding on type I hydrogels.

The preliminary biocompatibility studies clearly indicate that both types of scaffolds are excellent candidate materials for a variety of tissue engineering applications.

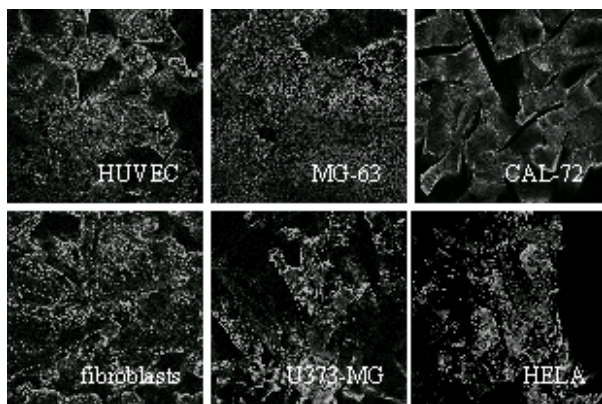


Figure 3: Visualisation (5X) of different cell types, 4 weeks after cell seeding on type II hydrogels.

Conclusion

We have shown by confocal microscopy that the porous gelatin scaffolds developed, supported the attachment and growth of human cells over longer time periods irrespective of their pore geometry and pore size.

Acknowledgement

Peter Dubruel would like to thank the Alexander von Humboldt Foundation for the financial support under the form of a granted Research Fellowship. The UGent authors would like to thank IWT-Flanders and the IUAP/PAI-V/03 for financial support.

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BIOCOMPATIBILITY OF NOVEL POLYESTER/HAP/TCP COMPOSITES

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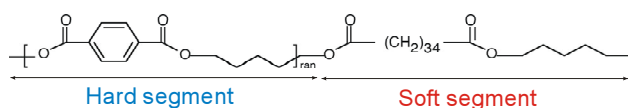
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Introduction

Elastomeric copolymers of poly(butylene terephthalate) (PBT) and dimmer fatty acid (DFA) have been evaluated as candidate materials for the preparation of tempory tendon prosthesis [1]. The exceptional properties of DFA, causing an excellent resistance to oxidative and thermal degradation, makes it possible to prepare these poly(aliphatic/aromatic-ester)s (PED) without phenolic, thermal stabilizers, a known irritant. For further improvement of biocompatibility, PED/ β -TCP composites were prepared [2]. These materials showed enhanced mechanical properties as well as apatite layer formation after exposure to 1.5 SBF for 28 days. In this work we report on biocompatibility of PED systems modified with HAP/TCP ceramic micropowders.

Materials and Methods

Poly(aliphatic/aromatic-ester) (PED) multiblock copolymer was synthesized according procedure reported earlier [1]. The chosen PED copolymer contains a very high amount of soft segments (70 wt%):



Composite material containing 5, 10 and 15 vol% of self synthesized HAP/ β -TCP (20:80 wt%) was prepared in a Brabender kneader at temperature of 150°C. The prepared mixtures were dried, palletised and afterwards compression moulded into polymer films 0.5 mm thick.

Cytocompatibility tests were carried out according ISO 10993-5. Embryo calvaria mouse MC3T3-E1 osteoblast-like cells (DSMZ, Braunschweig) with a density of 1×10^5 cells/ml were seeded for a 48 hours test. WST-1 test assay was used to estimate the mitochondrial activity of the cells. By trypsination enzymatically detached cells were counted with a cell counter. Results of four measurements on each material are presented as means $\pm$ SEM. Analysis of variance (one-way ANOVA) was performed. Cell morphology was examined with SEM.

Results

It has been found that increasing amount of inorganic phase within a polymer matrix contributed to enhanced increase in mitochondrial activity and the cell number was observed. With increasing amount of calcium phosphate cells show a better spreading on the composite material (Fig. 1). Biocompatibility of polymer/ceramic composites has also increased as compared to neat polymer.

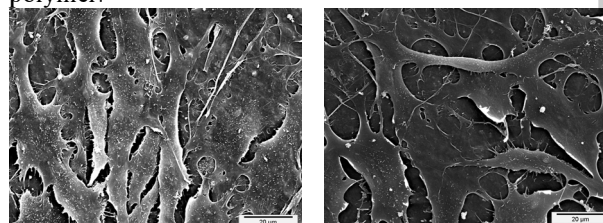


Fig. 1: SEM pictures of cells on material surface: PED+5 vol% (left) and PED+15 vol% HAP/ β -TCP (right)

HAP/TCP strongly affects polymers structure, as found from thermal analysis. An appearance of low temperature melting transition can be ascribed to phase transition of amorphous part of hard segments as result of strong interactions with ceramic microparticles (degree of crystallinity was also decreased with increasing amount of ceramic). It has been found that increasing amount of inorganic phase within a polymer matrix contributed to increasing mechanical properties with respect to elastic modulus, however tensile properties were decreased.

Conclusions

By adding HAP/TCP to the neat PED copolymer material

- phase morphology and mechanical properties are changing with increasing amount of ceramic
- *in vitro* cytocompatibility is enhanced for composite materials as compared to neat copolymer and increases with increasing amount of HAP/TCP.

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IN VITRO BACTERIAL COLONIZATION OF POLYMERIC IMPLANTS SURFACE

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Introduction

Adhesion of bacteria to human tissue surfaces and implanted biomedical surfaces is an important issue in pathogenesis of infection. It is known that certain strains of bacteria, particularly *Staphylococcus*, secrete extracellular polymeric substances after they adhere to implant surface, making it less accessible to the host defense system and significantly decreasing antibiotic activity [1]. They can remain non-active on the material surface for a long period of time until the environment allows them to overgrow (e.g. with decreased host immune function or poor tissue ingrowth around the prosthesis), and then clinical infection occurs. The number of methods and significant achievements are numerous, but the problem of total elimination of the infection is not solved. In a two stage flexor tendon reconstruction with a silicone rod, about 15% of complication is associated with infections [2]. In this work we investigated susceptibility to microbial colonization of novel polyester multiblock, a candidate material for temporary flexor tendon prosthesis with commercially available silicone implant (Hunter prosthesis) and polyurethane multiblock copolymer.

Materials and Methods

Poly(aliphatic/aromatic-ester) (PED) multiblock copolymer was synthesized according procedure reported in [3]. The PED copolymer is composed of a very high amount of soft segments (74 wt %), thus having elasticity and softness comparable to silicone rubber. Polymer thin films (0.5 mm) were immersed in 0.02% agar containing *Stag.* and MRSA of concentration of 0.5 McFarland (Vitek Systems ATB 1550). Samples were incubated at 37°C for 24 hours. Polymer samples were rinsed with 0.9% NaCl and fixed with 70% EtOH. SEM was used to evaluate polymer/bacteria surface morphology. Laser scanning citometry (LSC) was used for quantitative evaluation of adherent bacteria.

Commercially available silicone rubber (Hunter Prosthesis, Dow Corning) and polyurethane (Dow) were used as benchmark materials.

Results

After incubation with *St.a.* and MRSA bacteria, there are highly significant differences in bacteria number on PED copolymer surface and the reference materials (silicone elastomer, SIL and polyurethane, PU). PED copolymer showed relatively low susceptibility to colonization with *St.a.* as compared to SIL and PU, but was less resistant to MRSA as compared to SIL. Interestingly, PU showed higher number of MRSA adhered to its surface. Considering surface hydrophobicity it can be concluded that PED copolymer having water contact angle 98° is more resistant to *St.a.* adhesion as compared to silicone elastomer (water contact angle 111°). In contrary, hydrophilic surfaces are more susceptible to colonization with MRSA, where highest number of bacteria were found for PU (water contact angle 77°). An opposite behaviour was found for *St.a.* strain. High number of adherent bacteria was reflected by biofilm formation (multiply layers of bacteria were detected by SEM).

Conclusions

It has been demonstrated that tested polymeric materials (PED, SIL and PU) were colonized with *St.a.* and MRSA strains. These results indicated that PU and PED require sufficient surface modification to prevent possible infection of implants, especially after colonization with MRSA

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SCANNING ELECTRON MICROSCOPY INVESTIGATIONS OF THE EFFECTS BIOACTIVE SURFACES ON HUMAN CANCELLOUS BONE

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Introduction

Bioactive surface coatings utilising calcium phosphate (CaP) are widely used in coatings for both orthopaedic and dental implants [1,2]. The CaP coatings are fast resorbable and especially the CaP brushite are used for coating of implants as a matrix for nucleation and growth of new bone [3].

Materials and Methods

Clamps of c-p titanium were coated by vacuum plasma spray process with titanium powder (Figure 1 left), following electrochemically coated with a composite of CaP-phases brushite with less hydroxylapatite (BONIT[®], thickness 20 µm, Figure 1, right), the normal procedure of implant coating.

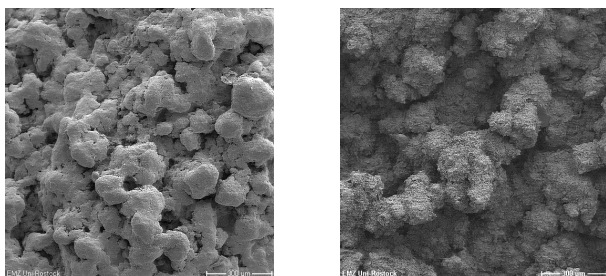


Figure 1: SEM images of the coated titanium clamps, with titanium powder (**left**) and following electrochemically coated with a composite of CaP-phases brushite (**right**).

In order to examine the sprouting of osteoblast cells on such surfaces, a defined piece of a human cancellous bone was clamped between the coated surfaces. The clamps with the cancellous bone were inserted onto tissue culture plates and were cultured in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal calf serum and with or without β - glycerophosphate at 37°C and in a 5% CO₂ atmosphere.

Results and Discussion

The contact area between cancellous bone and coated substrates are of special interests for our investigations. The current results of our in-vitro investigations demonstrate that bone cells revealed a well-spread morphology on the clamp surface after short time and the CaP coating was already resorbed (Figure 2-3). The morphology of the BONIT[®] coatings represents an ideal temporary matrix for bone regeneration.

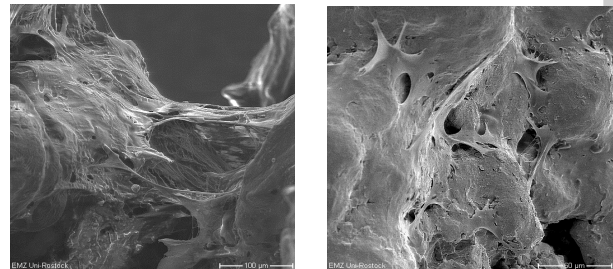


Figure 2: SEM images of the contact area between cancellous bone and coated titanium-surface (**left**), and outlying the contact area (**right**). Both surfaces were cultivated with cancellous bone cells for four weeks in DMEM with β - glycerophosphate

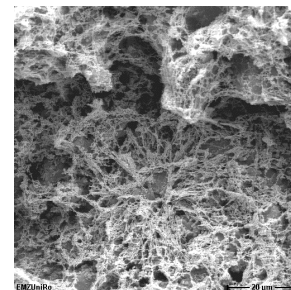


Figure 3: SEM images of sprouting cancellous bone cells after three weeks in DMEM without β - glycerophosphate.

Resuming investigations will concern themselves with the influence of the growth medium on cell population and the investigation of the cellular environment.

Conclusions

At present a high amount of research on coatings and biomaterials are conducted in animal models.

We assume the number of animal experiments required can be reduced using our in-vitro clamp system, i.e. native biological material in closely contact with the examined material.

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STRUCTURING OF COCHLEA IMPLANT SURFACES WITH FEMTOSECOND LASER PULSES

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Introduction

Recently, there is an increasing interest to provide different medical implants with functional surface structures. These structures should influence the attachment and growth of cells and therewith improve the effectiveness of medical implants. The search for optimal structures requires a flexible and cost-effective micromachining technology. Femtosecond lasers have been found suitable for processing of a wide range of materials with sub-micrometer resolution. Therefore, it is attractive to use this technique for surface structuring of implant surfaces. We present first test structures to investigate cell-surface interaction for improved cochlea implants.

Structuring of cochlea implant electrodes

The interaction of cochlea implant electrodes with neurites can interfere with growth of fibroblasts on the electrode surfaces. Providing these surfaces with structures repressing growth of fibroblasts is one possibility to improve signal transmission to the neurites. Using a femtosecond laser enables different techniques for machining of samples which can be used in fibroblasts growth experiments. Figure 1 shows an example of direct laser ablation. The sample is made of glass and can be sputtered with different metal films. Figure 2 shows an array of microbumps and nanojets on gold. Here, a direct-write ablation-free structuring technique based on laser-induced melt dynamics has been applied [1]. A third technique is shown in figure 3. Two-photon polymerization has been used for building up these cages [2].

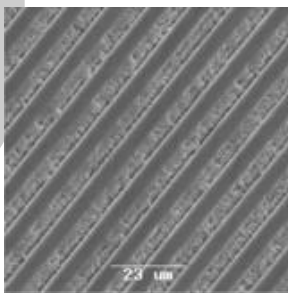


Figure 1: Scanning electron microscope (SEM) image of a gradient structure in glass fabricated by direct femtosecond laser ablation

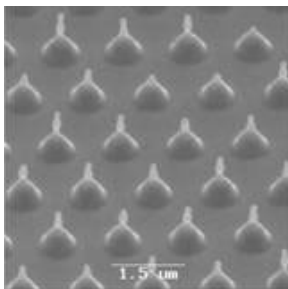


Figure 2: SEM image of an array of microbumps and nanojets on gold

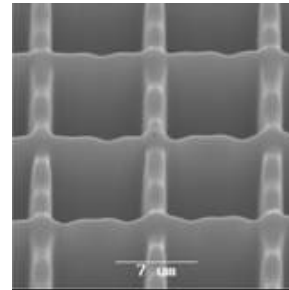


Figure 3: SEM image of a cage structure fabricated by two-photon polymerization

Structuring of the cochlea implant silicone surface

The carrier of a cochlea implant consists of silicone. Fibroblasts on the silicone surface can overgrow the small electrodes. To prevent this, repressing growth of fibroblasts even on the silicone surface is necessary. Unfortunately, direct laser structuring of silicone is ineligible, because silicone can become toxic due to collateral chemical changing. A solution to this problem is using a structured mould for curing the silicone. Figure 4 illustrates this. A femtosecond laser can be used for structuring the mould. All of the above mentioned laser structuring techniques can be applied.

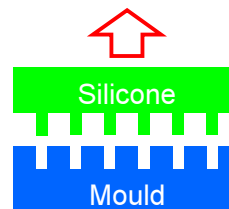


Figure 4: Structuring of silicone by curing in a (laser) structured mould

Conclusions

Femtosecond lasers are particularly suitable tools for surface structuring of medical implants. Structure sizes down to the sub-micrometer range are possible. Samples for cell adhesion tests can be fabricated with high flexibility in nearly all solid materials. Soft materials like silicone can be structured by curing in a laser structured mould.

Acknowledgements

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AN IN-VITRO STUDY OF PROTEINS AND PLATELETS INTERACTIONS WITH CARBON COATED MATERIALS FOR HEART VALVE IMPLANTS

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Introduction

Due to its good mechanical and biocompatibility properties, pyrolytic carbon is largely used as coating for biomedical devices. Since the first use of pyrolytic carbon for heart valves manufacturing, many attempts have been done to produce different types of biocompatible carbon materials. Nevertheless, there are relatively few studies of protein adsorption to low temperature pyrolytic carbon.

Aim of this study was to characterize two different types of carbon films for heart valves prostheses and to analyse the biological phenomena occurring at the interface with blood, such as protein adsorption and platelets adhesion.

Materials

Two different carbon coatings have been investigated: a 500 µm-thick silicon-alloyed pyrolytic carbon, (sample A, n=5), and a low-temperature carbon film deposited on Stellite (B, n=5) or on silicon (C, n=5). Each sample, was polished, pickled, cleaned and sterilized before testing. Formvar (polyvinyl formal)-coated glass slides (F, n=5) were used as controls.

Surface characterization of materials was performed by high-resolution scanning electron microscopy (ESEM-FEG XL30 FeiCompany), EDS microanalysis (EDAX), atomic force microscopy (AFM NT-MDT Smena; Solver PRO) and contact-angle goniometry.

In vitro biological tests were performed by incubating sample materials with platelets poor plasma (PPP, 5 min at 37°C) and platelets rich plasma (PRP, 15 min at 37°C). Mono-dimensional (1D) electrophoresis (NUPage® Novex 3-8% Tris-Acetate and 4-12% Bis-tris gels, Invitrogen, CA) and western blotting (Western Breeze® Chromogenic Western Blot Immunodetection Kit, Invitrogen, CA) were used to identify proteins adsorbed from human plasma, and to evaluate the binding strength of proteins to the surfaces. Number, morphology and activation degree of platelets adhered to sample surfaces were evaluated by SEM.

Results and Discussion

Equilibrium contact angles were similar for the different samples examined, evidencing an hydrophilic behaviour of the coatings. The hysteresis between advancing and receding contact angle (41, 48 and 39° for samples A, B and C, respectively) is index of high surface heterogeneity and seems to be related to surface chemistry and morphology.

A preferential adsorption of albumin from PPP was detected on sample A by the analysis of binding strength electrophoresis. Samples B and C presented, in addition to albumin, traces of proteins in the range 100-300 kDa weakly adsorbed to the surfaces. Formvar reference adsorbed high- and medium-molecular weight plasma proteins and released them within three elutions by water. Western Blotting enhanced the presence of fibrinogen eluted from all the different surfaces.

The analysis of PRP-incubated samples showed on sample A the smallest amount of adherent platelets (60 ± 24 on a surface of $11650 \mu\text{m}^2$) and a low degree of activation with a small percentage of spread platelets. On sample B the most platelets ($57 \pm 12\%$) assumed a spread conformation, while dendritic platelets were characterized by very long and expanded pseudopodia. Some aggregates ($<10 \mu\text{m}$ large) formed by 15-20 platelets were also present. Sample C presented a dominant percentage of dendritic adhering platelets.

The number of spread platelets was significantly higher on Formvar than on the other sample materials and a negligible amount of round platelets was detected on Formvar surfaces.

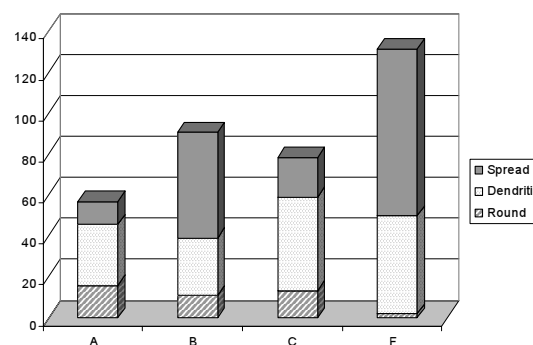


Figure 1: Count of platelets attached to sample surfaces and shape distribution (Round: not activated platelets, Dendritic: pseudopodia protracting to the surface, Spread: flat and stretched on the surface).

Conclusions

The interactions taking place at the interface between carbon-coated materials and blood were studied by the analysis of phenomena occurring during PPP and PRP contact. The results of this study showed a significant difference in the biological response of carbon materials with respect to Formvar reference and a partial influence of the substrate on haemocompatibility properties of the coatings.

CELL INTERACTION WITH PHOSPHOLIPID IMPLANT COATINGS

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Introduction

The use of metal implants is appropriate for a broad range of applications in bone repair or substitution. For a better acceptance of the implant, coating of the implant surface is one of the main interests in current research. One approach is the covering of the metal surface by phospholipids lipids (e.g. POPE) leading for example to an increase in chondroitin sulphate production by 30% for Human Articular Chondrocytes (HAC). Still a variety of other lipids is available for implant modification. Their effect on MG-63 cells, gram positive/negative bacteria and macrophages is evaluated.

Material

POPC (palmitoyl-oleoyl phosphatidyl-choline), known for the reduction of cell attachment of erythrocytes [1], POPE (PO phosphatidyl-ethanolamine), POPS (PO phosphatidyl-serine), known to be a signal for apoptosis and calcification [2], and POPG (PO phosphatidyl-glycerol) which originates from bacterial membranes, were solved in isopropanol:ethanol (50:50, 1mM). The solution was evaporated and dried over night on mirror polished Titanium, Ti-6Al-4V and Ti-6Al-7Nb.

The cellular interaction was tested with MG63 cells, RAW 264.7 mouse macrophages, gram negative (*E. coli*) and two strains of gram-positive bacteria (*Staphylococcus carnosus* and *Bacillus subtilis*). Cell adhesion was observed by SEM; viability measured by MTT and neutral red-assays. Immunogenic response of macrophages was analyzed by NO- and TNF- α measurements.

Results and Discussion

The analysis of bacterial interaction with phospholipids showed in general a significant reduction of adhesion in comparison with the uncovered metal surface, a positive fact in terms of possible infections.

The NO- and TNF- α production by macrophages showed a differentiated response depending on the lipid. POPS evoked a strong immunogenic reaction in RAW 264.7, leading to apoptosis. POPG also induced NO- and TNF- α production, but viability was higher than for POPS. On the contrary, POPE and POPC did not induce a measurable immunogenic reaction. These results are in good agreement with the literature, as PS is known to be a relevant lipid in apoptosis. Taking the prokaryotic origin of PG into account, the macrophage reaction to this lipid can easily be explained. POPE and POPC as

integral part of the eukaryotic cell membrane are not supposed to induce a cellular reaction in macrophages.

For MG63 cells, to be considered as a model system, apoptosis was induced on POPS (Fig. 1). The other lipids had a positive effect on cell attachment, which is quite intriguing for POPC, because the PC headgroup is used combination with polymers to reduce cellular adhesion on materials with blood contact (2).

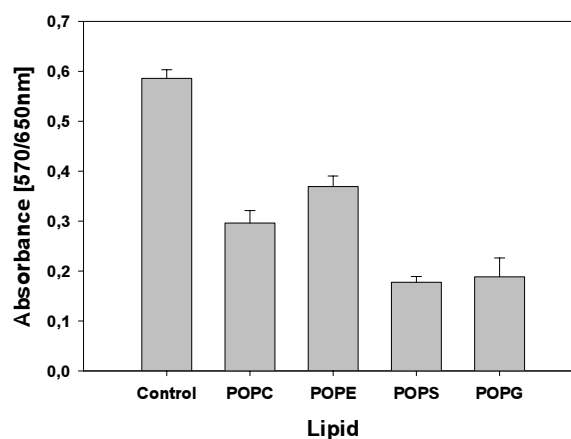


Figure 1: MTT-Assay of MG-63 cells

Conclusions

Coating of implant surfaces with phospholipids is easily achieved and leads – depending on the lipid – to a positive MG-63 interaction with the implant. The most promising lipids for the cell attachment and proliferation are POPC and POPE. These lipids prevent bacterial adhesion and do not induce inflammatory response of macrophages.

Acknowledgements

We gratefully acknowledge the donation of RAW 264.7 cells by A. Gasser and wish to thank Dr. J. Schnieders, Phillips University Marburg, for the donation of the osteosarcoma cell line.

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CHEMICAL FUNCTIONALIZATION OF TITANIUM SURFACES BY PLASMA ASSISTED POLYMERIZATION

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Introduction

Plasma polymerization is a useful tool for coating of metals with strongly fixed adhesion promoting layers. Chemical functional groups like amino-, hydroxyl-, carboxyl- or aldehyde groups act simultaneously as an anchor for the covalent coupling of linker- with biomolecules [1]. The aim is to provide a well defined chemical basis for better cell adhesion and spreading. First promising results could be obtained.

Materials and Methods

Polished titanium (grade 2, R_a 0.01 μm) was coated with a very thin layer of plasma polymerized allylamine (PAAM) using a pulsed low pressure microwave discharge plasma (2.45 GHz, 500 W, 50 Pa, 0.3 s/1.7 s on/off, 50 sccm Ar / 50 sccm AAm) [2]. The effective treatment time was 144 s. A polymer layer thickness below 100 nm could be obtained and characterised by different physicochemical analysis methods as XPS, FTIR and contact angle. Chemical coupling reactions of the plasma generated amino groups [3] with collagen 1 via different linker molecules as polyethylene glycol diacid (PEG-DA) or glutardialdehyde (GDA) could be realised.

Results and Discussion

The XPS surface analysis revealed a chemical composition of about N/C=25% and O/C=20% in the three different coupling reactions with collagen 1 (Fig.1). Except for the coupling reaction of PEG-DA a pinhole-free layer without a Ti signal could be observed. The peptide bond CO-NH characteristic for the collagen coupling could be proved at 288 eV (Fig.2).

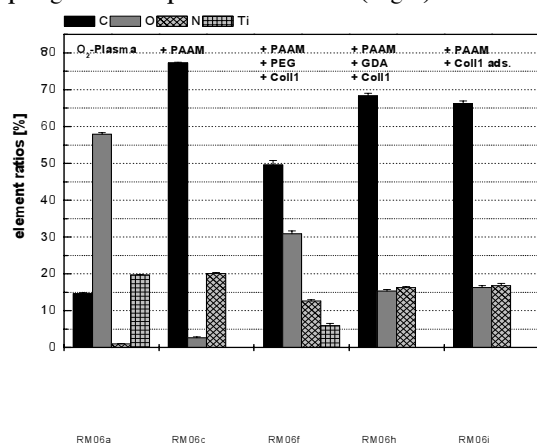


Figure 1: Element ratios of Ti-surface plasma treatments and following coupling reactions with different linker molecules

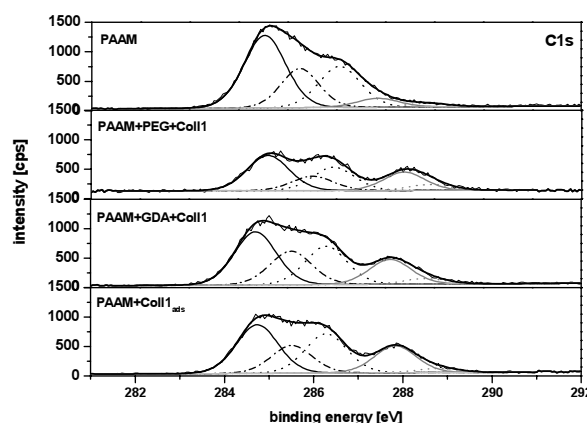


Figure 2: XPS results of the C1s spectra for coupling reactions of PAAM with collagen 1 via different linker molecules. The characteristic peptide bond CO-NH appears at 288 eV.

The investigation of initial cellular effects as adhesion and spreading of osteoblasts demonstrate the advantage of amino functionalized plasma polymerized titanium surfaces [4, 5].

Acknowledgements

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THE EFFECT OF RAPIDLY RESORBABLE BONE SUBSTITUTE MATERIALS ON OSTEOBLASTIC DIFFERENTIATION

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Introduction

Ideally, bioactive ceramics for use in bone regeneration should stimulate the differentiation of osteoprogenitor cells into osteoblasts at their surfaces in combination with a high degradation rate. Compared to the materials which are currently clinically available there is a considerable need for materials which resorb more rapidly but stimulate osteoblast differentiation at the same time. This has led to the development of novel calcium-alkali-orthophosphate ceramics with a higher solubility compared to tricalcium phosphate (TCP). Therefore, this study examines the solubility of these novel materials as well as their effect on the expression of an array of osteogenic markers by human osteoblast-like cells (SaOS-2) as compared to the currently clinically used material TCP.

Material and Methods

Discs were prepared for cell tests from calcium alkali phosphate (GB9 and GB9/25) granules as well as TCP. GB9 and GB9/25 contain the crystalline phase $\text{Ca}_2\text{KNa}(\text{PO}_4)_2$ and small portions of amorphous compounds, which differ in their quality and quantity. SaOS-2 human bone cells were grown on the substrata for 3, 7, 14 and 21 days, counted and probed for an array of osteogenic markers (type I collagen (Col I), alkaline phosphatase (ALP), osteopontin (OP), osteocalcin (OC), osteonectin (ON) and bone sialoprotein (BSP)) using a quantitative immunocytochemical assay. The solubility was determined in TRIS Puffer 0.1M (pH7.4, 37°C) by using particles according DIN ISO EN10993-14. For determining the long term solubility this procedure was repeated weekly for 12 weeks. The concentration of the leached ions was determined by ICP-OES.

Results and Discussion

In Fig. 1 the results of the long term solubility measurements are depicted. The calcium alkali phosphates clearly display a greater solubility than TCP. The high solubility of GB9 and GB9/25 is caused by the alkali leaching, but it takes much longer until the main solubility process occurs in GB9/25 compared to GB9. This phenomenon is probably caused by the differences in quality and quantity of the amorphous phase. These differences result from GB9/25 containing more P_2O_5 compared to GB9.

The different calcium phosphates tested also significantly affected cellular growth and the temporal expression of an array of osteogenic markers. But these differences did not have the same magnitude as those noted with respect to the solubility

measurements. Fig. 2 shows the numbers of SaOS-2 cells which were recorded at 3, 7, 14 and 21 days on the different bioceramics. GB9 had the most effect on proliferation and differentiation of SaOS-2 cells, inducing type I collagen formation as well as expression of osteopontin, bone sialoprotein and osteonectin protein at 3, 7 and 14 days indicating later osteoblast differentiation. A pattern which was maintained at a later time point (day 21) for Col I, ON and OC. OP, ON, OC and BSP have been tightly linked to osteoid production and matrix mineralization, suggesting that this material may possess a higher potency to promote osteogenesis and matrix calcification, than TCP and GB9/25.

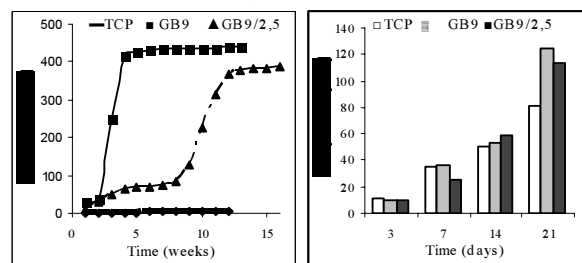


Fig. 1: Long term solubility behaviour of bioceramics

Fig. 2: Number of SaOS-2 cells cultured over 21 days on different bioceramics.

At 7 days, cells on GB9/25 displayed greater expression of Col I and BSP than cells on TCP. A pattern which was maintained at 14 days and accompanied by enhanced cellular proliferation compared to the TCP surfaces. Moreover, at 21d, similar to GB9, GB9/25 surfaced displayed greater cell numbers expressing higher protein levels of OP, ON, OC and BSP compared to cells on TCP. Since these osteoblastic markers characterize the later stages of osteoblast differentiation, this is suggestive that GB9/25 may also possess a higher potency to promote osteogenesis and matrix calcification than TCP.

Conclusions

In conclusion, both GB9 and GB9/25 displayed a considerably greater solubility as well as a greater stimulatory effect on osteoblastic proliferation and phenotype expression compared to TCP. This is indicative that GB9 and GB9/25 are excellent candidate bone substitute materials for promoting osteogenesis and bone regeneration.

Acknowledgements

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EFFECT OF MULTIFUNCTIONAL DICLOFENAC SODIUM RELEASING BIOABSORBABLE IMPLANT ON MACROPHAGES. AN IN VITRO STUDY.

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Introduction

So far, bioabsorbable polymeric implants have been developed and used for bone fixation. However, they are associated with inflammatory reactions that may lead to osteolysis. Combining the material with targeted long-term release of anti-inflammatory agents can reduce such complications. The objective of the current study was to evaluate *in vitro* the effect of diclofenac sodium releasing bioabsorbable polymer on macrophages.

Materials and Methods

Poly(lactide-co-glycolide) 80/20 (PLGA 80/20) was combined with diclofenac sodium in 2, 4 wt-% and extruded into rods using melt extrusion. Discs of 6 mm (diameter) and 3 mm thick were cut. Mouse macrophages (TIP-67) were cultured on top of the discs: 1) PLGA + 4 wt-% DS, 2) PLGA + 2 wt-%, 3) PLGA (posit. control). Macrophage culture without implants was also used to serve as negative control. Apoptosis was checked at 5, 6, 7 and 11 days *in vitro*.

Results and Discussion

Some reductions in apoptosis of MPs was seen in 4 wt-% DS containing samples. However, 2wt-% DS containing samples were associated with survival of more MPs than control PLGA.

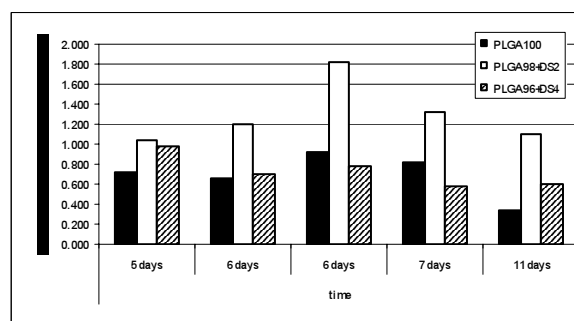


Figure 1. Cell numbers relative to negative control after 5, 6, 7 and 11 days culturing.

Conclusions

DS releasing implant containing 4 wt-% of DS may be associated with increased apoptosis of macrophages according to this pilot study. Such material would be useful for relief of foreign-body inflammatory reactions.

Acknowledgements

This work is in the framework of the European Commission Network of Excellence (EXPERTISSUES Project). Research funds from Technology Development Center in Finland (TEKES) are greatly appreciated.

MICRO STRUCTURING OF IMPLANT SURFACES BY PARTICLE FILLED SOL-GEL COATINGS AND CELL REACTION *IN VITRO*

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Introduction

Structuring surfaces in the μm -range by sandblasting is the state-of-the-art treatment for non-cemented hip implants and the cell-material interactions have been studied extensively *in vivo* and *in vitro* [1]. However, besides those macrostructures also micro- and even nano-structured surfaces have an effects on cell behavior and biocompatibility [2]. This paper describes a cost-effective method for structuring surfaces by adding powder particles to a biocompatible, sol-gel derived coating for implants and the influence of calcium ions released from those on tissue cells *in vitro*.

Materials and Methods

A suspension was prepared by mixing hydroxyapatite powder (Merck, Germany) with a dispersing agent into a titania sol [2] (0, 1, 3, 6, 8 wt%). The coating was formed by a dip-coating process on glass-discs and a subsequent heat treatment (500 °C). The *in vitro* cultivation was done in direct cell contact with MC3T3-E1 cells (48h, 37 °C, 5 % CO₂) in α -MEM (Life Technologies, UK). Additionally, 50 $\mu\text{g}/\text{ml}$ ascorbic acid, 10 mM β -glycerophosphate, 1 vol% penstrep (Sigma, Germany) and 5 % (v/v) fetal calf serum (FCS, Life Technologies, UK) were added. Cell number was measured with a cell counter (Coulter Z2, Beckman, Germany) and the mitochondrial activity by the WST-1-assay (Roche, Switzerland), respectively. The calcium release from the coatings was determined by the o-cresolphthalein complexone method (Sigma, Germany).

Results and Discussion

The ideal dispersing agent for the deagglomeration of HA-powder in the organic sol was evaluated through extensive test series. The resulting coating shows HA-particles (D₅₀: 0.3 μm) homogeneously distributed in a crack free titania matrix (Figure 1) with high adhesion strength to the substrates. The roughness (R_a) is about 0.5 microns. With rising powder content the surface roughness keeps almost constant, but the amount of calcium ions released from the particles under physiological conditions increases significantly (Table I).

Table I: Ca⁺²-Release from powder filled coatings with raising filler content.

HA-content [wt%]	0	1	3	6	8
Ca ²⁺ -Release [$\mu\text{g}/\text{l}$]	0.00 $\pm$ 0.00	0.17 $\pm$ 0.04	0.75 $\pm$ 0.06	1.87 $\pm$ 0.1	3.22 $\pm$ 0.68

Linked to this, the cell number and the mitochondrial activity of MC3T3-E1 cells also increase (Figure 2). At a filler content of 3 wt% a plateau seems to be reached.

Higher filler content doesn't change the cell behavior significantly.

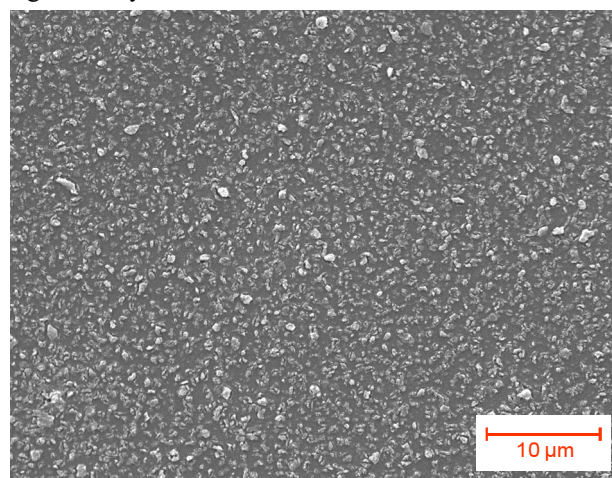


Figure 1: HA-particle-filled titania coating (R_a: 0.5 μm).

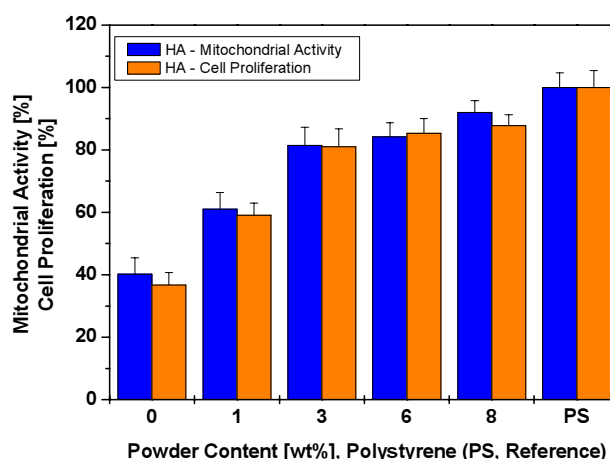


Figure 2: *in vitro* cell behavior on HA-particle filled titania coatings depending on the amount of particles.

Conclusion

Compared to a titania-coated alloy without powder filling, the cell number as well as the mitochondrial activity of tissue cells doubled by incorporating hydroxyapatite powder (3–8 wt%) into the coating.

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FIBRONECTIN ANCHORAGE STRENGTH CONTROLS ENDOTHELIAL CELL MORPHOLOGY

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Introduction

Angiogenesis, the formation of new blood vessels from endothelial cells plays a critical role in embryogenesis and wound repair. Furthermore, the success of tissue engineering strategies using artificial implants often depends on a well developed vascular network. That's why it is of great importance to control the interaction of the endothelial cells with artificial scaffold materials through the presentation of extra cellular matrix components on the surface of the scaffold material.

We investigated the influence of the anchorage strength of the extra cellular matrix protein fibronectin (FN) on the angiogenesis of human umbilical vein endothelial cells (HUVEC) on planar polymer substrates.

Materials

Thin layered coatings of alternating maleic anhydride copolymers (MA-copolymers) covalently linked to solid surfaces are used as a versatile platform for bio-surface engineering. By the choice of the comonomer the physicochemical characteristics of the copolymer coating can be adjusted in a very controlled way [1]. FN was bound with different anchorage strength (physisorption at surfaces with varied characteristics and covalent linkage) and in different amounts to the copolymer films. HUVECs grown in culture on these surfaces for 5 days were studied by fluorescence laser scanning microscopy, reflection interference contrast microscopy, and raster electron microscopy.

Results

The initial FN concentrations but also the protein anchorage strength of the MA-copolymers were found to influence the formation of extra cellular matrix by the cells as well as their morphology.

On surfaces with strong protein anchorage the cells exhibit a flat morphology [2] independent of the initial FN concentration. The reorganised FN network varies from nearly two-dimensional on surfaces with a high initial FN coverage to a coarse three-dimensional extra cellular matrix at low pre-coated FN amounts.

In contrast, on surfaces with weak protein anchorage strength the cells are able to form a three-dimensional FN network even with a high initial FN coverage [2].

However, with decreasing pre-coated FN amounts the complexity of the network decreases. The growth pattern of the cells was found to be clearly related to the FN pre-coating as well. With increasing the pre-coated amounts of FN the cells better adhere to the substrate surface reaching a closed cell sheet at very high concentrations. Interestingly, there exists an intermediate range of FN concentration, where the cells begin to form capillary like networks.

The initial immobilized FN amounts also influences the deposition of other matrix proteins, such as collagen IV and vitronectin. These two proteins were found to be assembled within existing FN networks only.

In the reported experiments cell adhesion to the extra cellular matrix is mediated by the $\alpha_5\beta_1$ integrin and, thus, by FN. Interestingly, the cells expressed the CD49e (α_5 chain of the integrin) independently of the FN amounts available demonstrating that the interfacial FN is sufficient to enable cell adhesion in any of the studied cases. However, differences are observed concerning the morphology of the HUVECs. This can be explained by the expression of the CD51 (α_v chain of the $\alpha_v\beta_3$ integrin [3]) only by the cells cultured on the surfaces with a strong protein attachment, where HUVECs are in closer contact to the surface.

Conclusion

The anchorage strength and the initial concentration of extra cellular matrix proteins to artificial surfaces were found to control the morphology of endothelial cells. This can be attributed to differences in the extra cellular matrix assembly as well as to the expression of different integrins. Surface characteristics can stimulate endothelial cells to form capillary networks on artificial materials.

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CELL RESPONSE TO Ti6Al4V-DISCS MODIFIED WITH TITANIUM OXIDE AND RGDS-PEPTIDES AND WITH DIFFERENT SURFACE ROUGHNESS

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Introduction

Biomaterials used for dentistry and orthopaedics need to have good physical, chemical and biological properties. Titanium and its alloys are widely used in medical applications, such as dental implants or hip-joint fixation. There are different methods to improve the biocompatibility of implant materials. One method is the coating with an inorganic layer [1,2]. Another method is the functionalisation of the surface with biomolecules to improve cell adhesion and cell proliferation. RGDS peptides were chosen for biofunctionalisation, which are known as a recognition sequence for cell membrane receptors (integrins) [3]. This paper deals with surfaces which have both an inorganic TiO₂-coating and an organic functionalisation on surfaces with different roughness. Murine mesenchymal MC3T3-E1 cells were cultured for 3 hours, 48 hours and 28 days on these modified discs. The different incubation times give information about cell adhesion and cell proliferation. Additionally, the gene expression (mRNA) of endothelial cells was compared between substrates with and without titanium oxide coatings.

Materials and Methods

Ti6Al4V discs were used as substrates, to which different grinding and polishing procedures were applied to create an array of surface topographies with differing roughness values.

A dense TiO₂-coating was generated on the discs by dip-coating via a sol-gel process and subsequent calcination at 500 °C. Additionally to this inorganic coating a bio-functionalisation was carried out. First, the ceramic surface was activated with 3-aminopropyltriethoxysilane. Then, 4-azido-1-fluoro-2-nitrobenzene was applied as spacer. Via UV-light, RGDS peptide was cross linked covalently to the spacer.

MC3T3-E1 osteoblast-like cells were cultured on coated and uncoated materials with different incubations times. Cell viability (WST-1 test) and cell number (BrDU) were analysed at the end of incubation.

Results and Discussion

The cell adhesion of MC3T3-E1 cells was determined by incubation in serum free medium over an incubation time of three hours. We used a serum free culture medium to get information about the direct interaction between the surface and the biological system. This way we can eliminate the influence of proteins from the culture

medium adsorbing to the surface. On Ti6Al4V and TiO₂-coated discs, an increase of cell number and cell viability with decreasing roughness could be seen. This trend was also found in cell experiments with 48 hours of incubation, which give information about the cytocompatibility of the differently modified materials. The same results were obtained from cell experiments over an incubation time of 28 days. However, when an additional RGDS-functionalisation is applied, this trend is lost for all incubation times. A higher cell number and cell viability was found on all functionalised samples, with similar values for the different degrees of surface roughness.

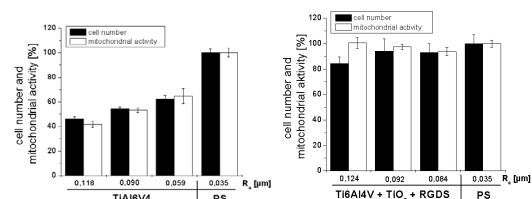


Figure 1: Results of cell tests with MC3T3-E1 cells cultured on Ti6Al4V discs with different roughness (left) and additional TiO₂-coating and RGDS immobilisation (right) for 48 h related to values on Polystyrol (100%).

Conclusions

The results can be interpreted thus that an RGDS-functionalisation has a higher influence on cell behaviour than surface topography. This could be useful for the optimisation of rough implant materials that are implanted cement-free.

Acknowledgements

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BISPHOSPHONATE RELEASING MULTIFUNCTIONAL IMPLANT

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Introduction

Bisphosphonates are used in inhibition of bone resorption, which is associated with bioabsorbable implants. The aim of this work was to develop and characterize bisphosphonate releasing bioabsorbable material.

Materials and Methods

Modified poly(glycolide-co-lactide) (mPLGA) 80/20 was compounded with clodronate disodium salt (CS) using melt extrusion. The billets were self reinforced (SR) and gamma sterilized. Four different implant types were investigated: A) SR PLGA 8 wt-% CS, B) sterilized sSR PLGA 8 wt-% CS, C) compounded PLGA 8 wt-% and D) sterilized sSR PLGA (control). Drug release was measured using UV-VIS spectrophotometer and mechanical properties were measured for groups B and D specimens during eight weeks in vitro.

Results and Discussion

After sharp jump start peak the rate of drug release from SR implants decreased to over

50µg/day 35 days. After start peak the release rate of compounded implants was near to zero for 70 days. Self-reinforcement and sterilization accelerated bisphosphonate release. Sterilized PLGA implants maintained their mechanical properties until four weeks. After eight weeks the shear strength was reduced 35% (82 MPa from the initial 128 MPa) (Fig. 2) and sSR PLGA with CS 124 MPa to 88 MPa.

Conclusions

It is possible to manufacture clodronate releasing PLGA rods by melt extrusion with regular drug release, with good mechanical properties.

Acknowledgement

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INTERACTION OF SKELETAL CELLS WITH DIFFERENT POROUS TITANIUM ALLOY IMPLANT SURFACES

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Introduction

Permanent implant materials are successfully used for the treatment of bone failure. A key role in acceptance of the implants plays the adhesive interactions between the implant surface and the cells of surrounding tissue. One possibility of surface modification of titanium-based alloy implants is the design of a porous metal structure on a solid implant core. The porosity of implant surface leads first to increase of surface area. Additionally tunnels and cavities are developed into which the cells could invade and form matrix. For this reason a bone ingrowth may occur and a tight integration of the implant into the surrounding bone can be achieved.

Material

Three different types of porous materials were manufactured by powder metallurgical routes. The first group based on Ti-6Al-4V was produced according to the Low Density Core (LDC) process. The second group, contains protein foamed porous samples, have been prepared by mixing viscous slurries consisting of metal powders (Ti-6Al-4V and Ti-6Al-7Nb, size fraction < 45 µm), protein, water and small amount of a dispersing agent. The third group of samples surface were obtained by sintering six different powder particle size fractions of Ti-6Al-7Nb powder (size fraction ranging from 75-500 µm) on mirror polished discs. For the most compact LDC specimen, micro tomography (µCT) elucidated the three dimensional structure. Pore sizes were obtained by using digital image analysis with AnalySIS from scanning electron microscopy (SEM) images and correlated to the cellular interaction, which was tested with human bone derived cells (HBDC), which were isolated from patients undergoing total hip arthroplasty and an osteosarcoma cell line MG63. Cell adhesion was observed by SEM; viability measured by MTT-assay.

Results and Discussion

Three different approaches to achieve a porous metal surface with connected pores were tested. µCT showed that the unconnected porosity of the LDC specimen ranges from 30-40% with pores sizes of 10-200 µm (Figure 1). The other two approaches led to samples with a higher connected porosity and pores sizes from 50 -

170 µm which can be designed depending on the initial powder particle size.

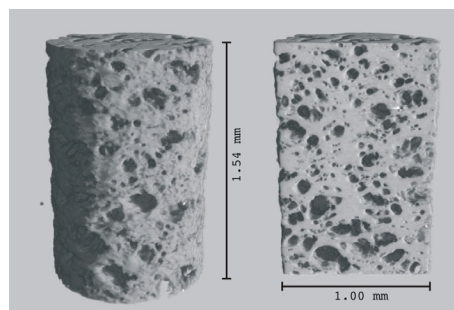


Figure 1: Micro tomography image of porous Ti-6Al-4V specimen produced by Low Density Core process.

The MTT-tests revealed that, in general, the number of adhered cells on the porous metal specimen is 50-80% of the cell number found on tissue culture plastic. Among the surfaces the cells prefer the sintered material. For HBDC-cells a linear correlation between pore size and cell viability was detected. The best results were found for pore sizes larger than 100 µm. MG63-cells do not show a systematic variation.

Conclusions

Sintered Ti-6Al-7Nb surfaces with pore sizes larger than 100 µm are most suitable for cell adhesion and viability for the osteosarcoma cell line MG63 and human bone derived cells. The design of this optimal porosity is possible by the appropriate powder particle fraction. Future measurements of the differentiation capacity correlated with the achieved porosity will elucidate, if the presented porous surfaces are promising candidates for the design of hybrid metal materials to improve osseointegration.

Acknowledgements

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MONITORING OF CELL BEHAVIOUR ON DIFFERENTLY STRUCTURED AREAS

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Introduction

Cells contacting an implant are affected by the implant surface structure. Latter interaction between cell and implant surface is an important key feature that determines the clinical success of implants. Beside the state of differentiation also the cell parameters cell shape, cell orientation, migration direction and migration velocity have been affected. The observation of cell behaviour, especially cell migration is therefore of key importance in order to understand the mechanisms of cell-material interactions and to predict cell performance on implant surfaces.

Materials and Methods

For observation of cell behaviour (3T3 mouse fibroblast cells), cells were prior stained with a fluorescent lipophilic dye (DiI) and seeded on a disc with differently structured areas. Thereafter, the structured surface with the attached fluorescent-labelled cells was transferred in an incubation chamber with a cover glass lid, which allowed long-term observations of the cell behaviour. Cell behaviour was monitored for several days by taking pictures each 15 minutes from the same previously selected areas of interest. Based on the migration pathway (trajectory), cell shape, migration direction and migration velocity were calculated by special image analysis software. Cell migration was analyzed on a titanium alloy disc with a plane surface and 10 differently structured areas. The cell behaviour was monitored on three different types of areas (V-shaped grooves, U-shaped grooves and $\cap$ -shaped elevations). The structures had a depth/height of 5 μm , 10 μm and 22 μm .

Results

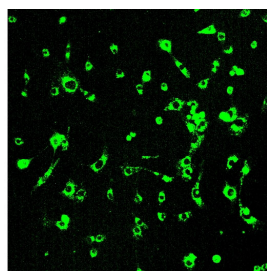


Fig. 1a

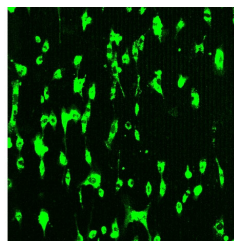


Fig. 1b

Fig.1: Migrating DiI-labelled cells. On the plane surface cells were not oriented (Fig. 1a), whereas on grooved surfaces the cells oriented along the axes of the grooves (Fig 1b). Further on the unstructured plane surface cells

with a round form were more frequently present than on the structured surfaces.

Cell shape: The cell shape with a length/width ratio of 1.1 to 1.5 was the most frequent type of cell shape on all investigated areas. Different types of cell shapes have been found on the differently structured areas. On the unstructured plane surface cells with a round form were more frequently present than on the structured surfaces (Fig. 1), whereas on the structured surfaces elongated cell forms were more frequent.

Cell orientation: Cells plated on the plane unstructured surface had no preferred orientation. In contrast, cells showed a significant preferred orientation on all structured surfaces in a sector $\pm 10^\circ$ parallel to the grooves/ridges. This was even true for structures where the tracks had a width of 30 μm . Only minor differences between the various types of structures had been found.

Cell migration direction: Cells plated on a plane surface migrate evenly in all directions. The covered trails of the cells migrating on the plane surface were random. In contrast, on structured surfaces cell migration occurred preferably in the two directions parallel to the grooves/ridges. The lowest frequencies of cell migrating in the direction parallel to the structures had been found on grooves with low depths respectively on structures with low elevations (5 μm). The effects of the structures on cell migration direction were much more prominent compared to the effects on cell orientation.

Migration velocity: Each cell changed its migration velocity continuously during the observation period. The mean cell migration velocity differed on the various surface structures. On structures with large widths (30 μm) cells exhibited significantly the highest migration velocity. A mean migration speed similar to the one found for cells migrating on the unstructured plane surface was found for most other structured surfaces with smaller widths. The migration velocity perpendicularly to the grooves/ridges was strongly inhibited. The degree of inhibition was different on the various structures.

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TITANIUM PROTECTION OF OSTEOBLASTS FROM OXIDATIVE STRESS INDUCED BY HYDROGEN PEROXIDE EXPOSURE

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Introduction

Titanium is a successful biomaterial used for many applications that possesses good biocompatibility and mechanical strength. It is covered by a surface layer of titanium dioxide. Mechanical disintegration of this stable inert oxide layer leads to fast reformation of titanium dioxide (corrosion). For that, the oxidation of titanium (anodic reaction) as well as the reduction of oxygen (cathodic reaction) is necessary. During the cathodic reaction, at least intermediately, reactive oxygen species (ROS) can be formed, which might affect interactions between titanium implant and surrounding tissue. The surrounding cells can be activated firstly transiently and production of reactive oxygen species by cells themselves can be induced. This ongoing process can cause later on permanent damage of cells and tissue and could be a reason for aseptic loosening of implants. In this study, we simulate oxidative stress by hydrogen peroxide and investigated effects on osteoblasts cultured on either titanium (Ti) or polystyrene (PS).

Material

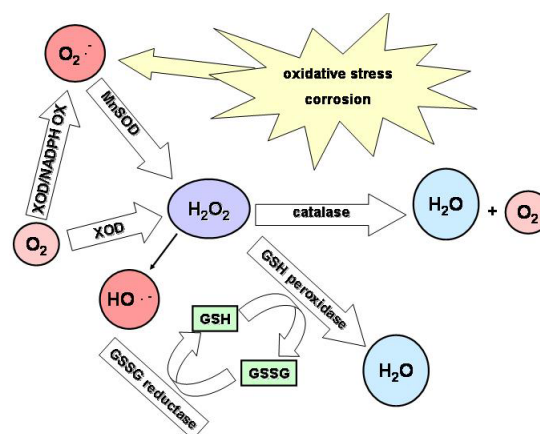
Osteoblasts were plated on polystyrene and titanium disc at a density of 25,000 cells/cm². 24 h after plating, different concentrations of hydrogen peroxide were added and cells incubated for different time points. The concentration of reduced glutathione was assessed by monochlorobimane in presence of glutathione-S-transferase. Expression and protein content of enzymes of oxidative defence, e. g. Mn-dependent superoxide dismutase (MnSOD), glutathione peroxidase (GPX), glutathione reductase (GSR), and catalase, were determined by real time PCR and Western blot analysis. SOD activity as well as metabolic activity and viability were also determined.

Results and Discussion

Hydrogen peroxide induces in osteoblast cultured either on polystyrene or on titanium a dose dependent decrease in metabolic activity as well as in viability. Actual H₂O₂ concentration in the culture medium was found to be reduced. Beside chemical decomposition of H₂O₂ are more or less active, cellular processes take place. One possible cellular pathway for H₂O₂ reduction is via direct scavenging of H₂O₂ by reduced glutathione (GSH) which works as a cosubstrate for glutathione peroxidase (Schema 1). The used reduced glutathione can be restored by activity of glutathione reductase. We found elevated GPX mRNA and GSR mRNA levels in

osteoblasts treated with H₂O₂ as well as higher amount of GSH in cells cultured on Ti.

Other possible pathway for H₂O₂ reduction is via catalase decomposition of H₂O₂. We found no induced expression of the catalase gene but increased protein level with Ti samples at 4h. In osteoblasts cultured on polystyrene, catalase protein level was increasing 6h after treatment. While also other reactive oxygen species could appear intermediately during H₂O₂ treatment, we investigated pathway in which superoxide dismutase (SOD) plays its role. SOD converts superoxide anion radicals produced in the body to hydrogen peroxide, thereby reducing the likelihood of superoxide anion interacting with nitric oxide to form reactive peroxynitrite. We found elevated levels of MnSOD mRNA in Ti samples at 1h followed by elevated mRNAs in samples on each material at 6h time point. Moreover increased SOD activity was determined.



Schema 1 oxidative stress pathways

Conclusion

H₂O₂ treatment of osteoblasts, in order to simulate corrosion of implants and ROS exposition of bone forming cells, induces glutathione as well as expression of glutathione peroxidase and glutathione reductase. Both H₂O₂ decomposing catalase and H₂O₂ forming, superoxide radical decomposing MnSOD are induced by H₂O₂. Osteoblasts cultured on titanium samples are more active in anti-oxidative defence in response to H₂O₂ compared to cells cultured on polystyrene. From this a protective effect of titanium could be assumed.

BIOMIMETIC MATERIAL COATINGS USING THE LAYER-BY-LAYER TECHNIQUE

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Introduction

Improvement of the biocompatibility is one of the main objectives in biomedical materials engineering. In order to attain a good tissue-implant-integration one approach is to create biomimetic surfaces to imitate the natural environment of mammalian cells, i.e. the extracellular matrix (ECM). Biomolecules such as adhesive proteins, growth factors, glycosaminoglycans (GAGs), etc. are suitable as they are ECM components of all tissues and mediate cell adhesion, proliferation and differentiation on biomaterials^[1-3]. The Layer-by-Layer technique (LbL) is a very comfortable and inexpensive way to attach such molecules. In principle, this method is a self-assembly process to modify charged surfaces independently of their 3D structure or morphology. It can be obtained by alternating adsorption of oppositely charged polyelectrolytes from aqueous solutions to a substrate^[4-6]. Chemical, morphological and mechanical properties of the coating can be controlled adjusting process parameters such as solution pH value, ionic strength and nature of the polyions, etc.

Materials and Methods

We applied here the polycations poly (ethylene imine) (PEI) and chitosan, a biological and biodegradable polysaccharide. As polyanion heparin, a GAG with anticoagulant activity was used to produce polyelectrolyte multilayers (PEMs) of different composition. The physico-chemical characterization included the study of surface wettability, adsorption regime, layer thickness and surface charge, resp. Water contact angle measurement, quartz crystal microbalance (QCM), ellipsometry and zeta-potential measurement were utilized. Furthermore, protein adsorption, cell adhesion and proliferation were investigated using the adhesive protein fibronectin (FN) and human fibroblasts, resp.

Results and Discussion

It could be revealed that the variation of the pH values as well as the ionic strength of the polyelectrolyte solutions influence the multilayer formation. On PEI/heparin PEMs both FN adsorption and cell culture tests in the presence of FN or serum indicated higher FN adsorption, cell attachment and proliferation on surfaces terminated by the polycation compared to the polyanion (see Fig. 1). This observation is supported by an enhanced cell adhesion on chitosan terminated PEI/heparin/chitosan PEMs. Probable causes are an electrostatic repulsion between the surface

and proteins and suboptimal conformation of heparin and/or FN under the applied process conditions. The targeted specific interaction of heparin with distinct binding domains of fibronectin (FN) could therefore not be demonstrated so far.

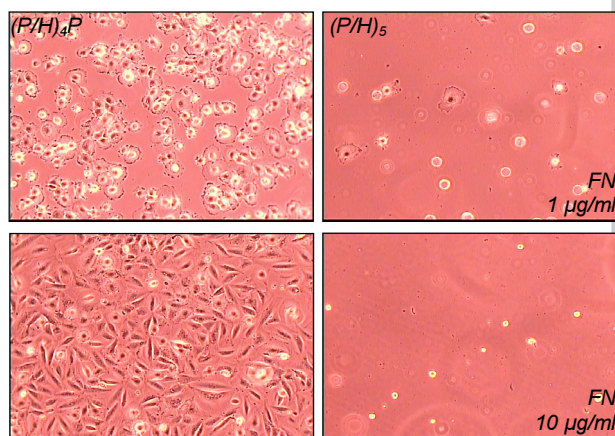


Figure 1: Cell adhesion after 4 h is reduced on heparin-terminated PEI/heparin PEMs (right column) but increased on PEI-terminated PEMs (left column) in dependence on FN concentration during preadsorption under serum free conditions.

Conclusion

In conclusion, the LbL assembly is a versatile method to coat the surface of diverse materials for tissue engineering application as well as implants^[4, 7]. Future work will be focusing the adjustment of process parameters, esp. the pH value of the utilized polyelectrolytes and proteins to achieve a targeted interaction including growth factors. Additionally, methods to analyze the morphology and mechanical properties of the PEMs will be employed.

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A NEW SYNTHETIC BONE REPLACEMENT MATERIAL WITH OSTEOINDUCTIVE PROPERTIES - IN VIVO INVESTIGATIONS

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Introduction

Hydroxyapatite (HA) being the main component of bone is an important material used for bone substitutes. Conventional HA ceramics are osteoconductive, but poorly degradable. A new HA matrix is presented being highly osteoconductive and at the same time fully biodegradable. Ectopic bone formation was induced when implanted subcutaneously into fatty tissue proving the osteoinductive properties of the new material.

Characteristics

The bone replacement material employed in this study was a HA scaffold produced in a sol-gel process at a temperature of < 700 °C. Evaporation led to the formation of small pores (Ø 5-100µm). The pores allow invasion of osteoblasts and connective tissue fibrils. The crystallites are loosely packed and held together by SiO₂ connecting the HA crystals and leading to nano-pores (10-20 nm). The extremely surface of the HA matrix (84m²/g) allows the adhesion of endogenous proteins inclusive growth factors (BMP). This interconnective porosity is the most important property of this new material. The proportional porosity of the granules amounts to 61% (density: 0.4-0.7 g/cm³, compressive strength: 40MPas) [1].

Osteoconductivity and biodegradation

In vivo studies revealed impressive bone formation within the HA matrix as early as 5 weeks after implantation. 8 months after implantation, the bone defects were almost completely (>96%) filled with regular bone. The HA matrix stimulated bone formation and at the same time was biodegraded by osteoclasts by way of bone remodeling. Histometrically less than 4% of the original HA matrix applied were found 8 months post-operatively. This favourable biological behaviour of the new HA matrix is due to the fact that the natural nanocrystalline structure and the highly interconnecting porosity is maintained during the sol-gel process. The SiO₂ component (amounting to 24%) additionally enhances bone formation [2].

Osteoinductivity

Osteoinductive means that a material induces bone formation outside the skeleton. The osteoinductive properties of the HA matrix were examined in adult Goettingen minipigs: In 9 animals 0.1 ml of HA matrix granules (0.6x2 mm) were applied into the subcutaneous

fatty tissue and adjacent musculature in the neck region. The tissue was excised after 2.5 and 8 months. The tissue samples were studied macroscopically, radiologically (CT produced better information) and microscopically. After 2.5 months (n=3) in all animals small foci of ossification were found histologically. Biodegradation of the HA matrix was considerably slower in soft tissues than in bone. After 8 months (n=6) in three animals multiple macroscopically visible foci with a diameter of 2.5 – 6 mm were found. The largest was 3.5 mm wide and 12.5 mm long. Smaller foci of ossification were found histologically only in two animals, while in one animal no extra-skeletal bone formation was verified. The close contact between partially biodegraded HA particles and newly formed bone spoke in favour of osteoinductive properties of the new bone replacement material. They were located almost exclusively in the subcutaneous fatty tissue. In only one animal an intramuscular micro-focus of ossification was found

Concluding Remarks

These studies prove that differentiation of adipocytes into osteoblasts can be induced in vivo by biomaterials with adequate structure without application of osteoinductive substances. Obviously muscular cells have almost lost this capacity – or there are less adult stem cells. Further in vivo studies will be required to understand those processes. The HA matrix is also a favourable carrier for other osteoinductive materials like bone morphogenic proteins and stem cells.

Clinical appearance

The HA matrix tested is available in form of granules for application in medicine (NanoBone®, Artoss GmbH, Rostock / Germany). First positive results in oral and maxillofacial surgery, i.e. in treating bone defects with the new HA matrix, have been published [3].

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THE NEW ART OF BONE GRAFT SUBSTITUTE DESIGN

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Introduction

A new method of design and manufacturing of bone graft substitutes is introduced. For the first time it is possible to prepare bone graft substitutes with a directed and controlled pore structure. Furthermore, the formation of complicated geometries is possible. First in vitro investigations with cell cultures show a vital cell growing deep within the synthetic bone graft material. Numerous applications are possible.

Materials

The desired type of synthetic bone graft consists of a mixture of calcium phosphate compounds embedded in an open-pored network of silica. According to the manufacturing process the material is characterised by a distinct pore distribution which can be controlled by varying the manufacturing conditions.

The new idea is to utilize freeze casting to form the porous silica network from an liquid sol. In such a manner it is possible to avoid the drying cracks arising during the usual xerogel preparation [1] which prevent the reliable formation of volume bodies. During the freeze casting process the sol is rapidly dehydrated by the local formation of ice crystals and thereby transformed to a silica gel network which becomes porous after freeze drying. Within the network, aside from calciumphosphates, several additional anorganic and organic components like trace elements and even cells can be incorporated. The spacial distribution of these additives is nearly homogeneous.

Results and Discussion

With the described method it was possible to prepare bone grafts with sophisticated geometric structures of varying pore size distributions. Several test preparations with different calcium phosphate components and mixtures of them were performed and led to error free compact bone graft substitutes. Experiments with additional organic polymers and incorporated reinforcing elements were also successful. The stability of the bone graft substitute is influenced by the distributions of pore size and pore alignment. Manufactured with a high amount of small pores the bone graft is more stable than with larger pores of less number density.

First tests with in vitro cell cultures indicate a vital cell growing even in deep seated regions of the bone graft substitute.

Conclusions

A new promising bone graft material prepared by a new versatile method is introduced. The first results predict a broad range of applications e.g. void filling, scaffold functions and restoring of large scale bone defects. The possibility to control the pore distribution both the size and the geometric alignment offer new choices for special adapted designs.

Further investigations are necessary to refine the bone graft substitute preparation according to resorbable reinforcing elements.



Figure 1: Different geometries of bone graft substitutes; an incorporated reinforcing metallic structure can be seen within the tube bone graft substitute (see center)

Acknowledgements

The REM investigations were performed at the Center of Electron Microscopy of the University of Rostock.

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LEUKOCYTES IN BIOMATERIAL INDUCED THROMBUS FORMATION

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Introduction

Biomaterial induced thrombus formation is an important draw-back of many blood-contacting devices as e.g. guide-wires, stents, artificial blood vessels, etc. Rapidly after first contact with blood, plasma proteins will adsorb to the surface and coagulation is triggered, resulting in thrombus formation [1].

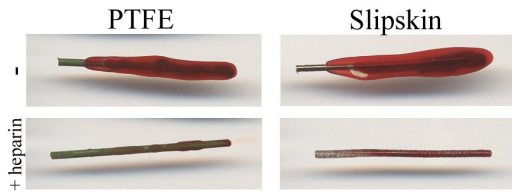


Figure 1: Pieces of guide-wire incubated in whole blood. Teflon coated (PTFE) and coils with a hydrogel coating demonstrate surface induced thrombus formation that can be inhibited by incorporating heparin in the coating

Also adhesion and activation of platelets are critical for surface induced coagulation [1]. The role of the red and white blood cells in thrombus formation however is less well understood. Hong and co-workers demonstrated that erythrocytes can influence thrombus formation on artificial surfaces [2]. The leukocytes (white cells) in the blood, are mainly involved in the immune response, and will rapidly bind to the surface of an implant. These bound leukocytes will subsequently release cytokines and chemokines, resulting in an inflammatory response. It has been proposed that coagulation can also be described as an inflammatory response of the blood [1]. The mechanism by which leukocytes influence biomaterial induced thrombus formation is not well elicited. Here we present that leukocytes play an important role in thrombus formation on artificial surfaces and that strategies to prevent leukocyte binding and/or activation may reduce thrombus formation on blood-contacting devices.

Materials and Methods

Blood was drawn from healthy donors and from this, platelet-rich-plasma (PRP), platelet-poor-plasma (PPP), and leukocytes were isolated. As model biomaterial we used pieces of guide wire, constructed from thin metallic wires coated with polytetrafluoroethylene (PTFE) or a hydrogel coating called Slipskin, that consists of n-vinylpyrrolidinone and butylmethacrylate (NVP and BMA)[3]. Coatings

with and without included heparin were studied. Thrombin formation in the absence and presence of leukocytes was studied in dynamic and static experiments. Also leukocyte adhesion and activation of leukocytes was studied.

Results

Thrombus formation of PRP on PTFE coils was accelerated when leukocytes were added (fig 2).

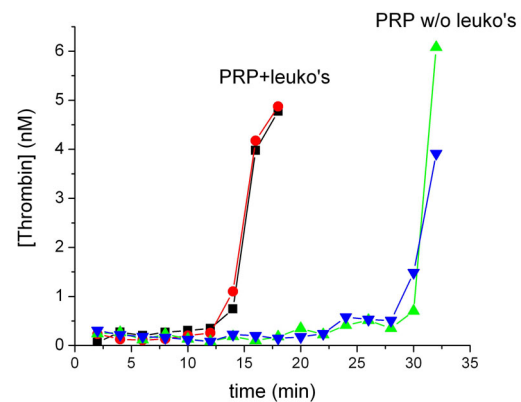


Figure 2: Thrombin formation under static conditions on PTFE surfaces in PRP and PRP with leukocytes added.

Leukocytes also bound to the surface of guide wires within minutes. Surprisingly, adding heparin to the coatings increased leukocyte binding. The bound leukocytes were found to expose active tissue factor on their surface, while non-bound leukocytes did not. Since tissue factor is a potent activator of thrombus formation in native vessels, this may be the mechanism by which leukocytes enhance biomaterial induced thrombus formation.

Conclusion

Leukocytes play an important role in thrombus formation on artificial surfaces. Leukocytes rapidly bind and expose tissue factor, inducing the formation of thrombin, and subsequent thrombus formation.

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POLYMERS WITH TUNABLE TOXICITY

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Introduction

Determining biocompatibility is a critical step in development of medical implants. Medical implants have to be biocompatible, meaning that they perform inside the body with an appropriate response in a specific application for the desired period of time [1]. Often biocompatibility is confused with biosafety, when only toxicity or viability is studied. Genuine biocompatibility can only be determined *in vivo*, but reliable *in vitro* assays with predictive value are required. A frequent problem faced during *in vitro* and *in vivo* compatibility testing is that materials display intermediate response (between non-toxic and highly toxic). The question then arising is: what can be interpreted as a compatible and what is a non-compatible response. The answer strongly depends on comparison to fully inert (e.g. high molecular weight poly-ethylene) or highly toxic (e.g. tin stabilized PVC) materials. The lack of a scale of materials with defined toxicity is hampering the objective interpretation of bio- and cyto-compatibility testing. For this purpose we designed a series of copolymers that display tunable toxicity. We demonstrate that the toxicity of leachables as well as the copolymer surface can be tuned. Then proliferation, morphology, and adhesion of cells in direct contact with the scale of materials were studied. This scale of materials is an important tool to device objective, quantitative bio- and/or cyto-compatibility assays.

Materials and Methods

Dimethylamino-ethyl-methacrylate (DMAEMA or D) and methyl-methacrylate (MMA or M) were distilled and co-polymerized in different molar ratios (D:M from 1:1 to 1:10). Control materials were pMMA and glass cover slips. 3T3 mouse fibroblasts, human endothelial cells and osteoblasts were used for this study. Cytotoxicity of leachables was determined using a standard MTT assay. Viability of cells in direct contact with the co-polymers was measured using the Live/Dead kit (Molecular Probes). Proliferation was determined using the marker Ki-67 and by measuring a proliferation curve of cells on disks. Cell morphology was visualized by staining the cytoskeleton with TRITC-Phalloidin, adhesion points by staining with an anti-phosphotyrosin or anti-vinculin antibody.

Results

Toxicity of both leachables and the washed non-leaching surface of the copolymers was determined. Increased D:M molar ratio results in higher toxicity of leachables (fig 1).

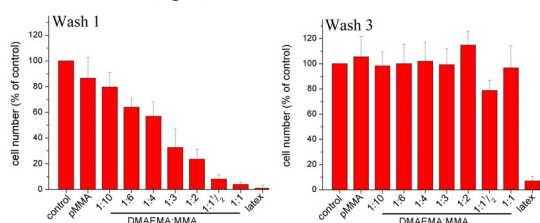


Figure 1: Cytotoxicity of leachables of the range of copolymers.

These leachables however, were washed out of the co-polymer after 2 washes. All subsequent direct contact assays were performed with thoroughly washed co-polymer disks. For the cells in direct contact with the materials, increasing DMAEMA content resulted in reduced viability, rounding of the cells (fig 2), a dramatic decrease in proliferation, and loss of focal adhesion points. Remarkably this loss of focal contacts was accompanied by an up-regulation of the focal adhesion proteins paxillin and vinculin.

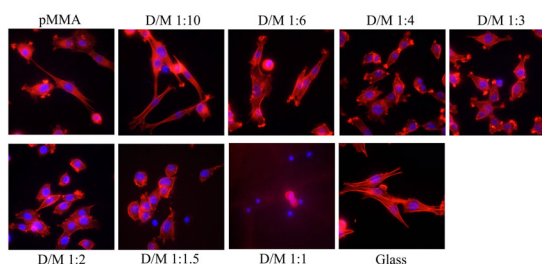


Figure 2: 3T3 cells on co-polymer disks stained with TRITC-Phalloidin (red) to visualize F-actin and DAPI (blue) to visualize the cell nucleus.

Conclusion

This set of co-polymers may prove a useful tool to study the behavior of cells on toxic materials *in vitro* and for the development of quantitative bio-and/or cyto-compatibility assays. The ability to tune the toxicity of the materials might make these materials suitable for a cytotoxicity scale.

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BIOCOMPATIBILITY OF A NEW ALUMINA PARTICLE FREE TITANIUM SURFACE

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Introduction

For more than 20 years alumina-blasted surfaces have been successfully used for cementless surgical implants, because of their good biocompatibility and rapid osseointegration. However, it has been shown recently that grit-blasting leads to partial embedding of aluminum oxide particles that can cover 24% to 40% of the surface, with a difference between manufacturers [1-2]. These particles can invade the joint space when dislodged and result in third body wear of the articulating surface [3]. To reduce this particle contamination, we developed a new surface treatment that is able to reduce these alumina particles to 1% to 6%, depending on the analysis method, without changing the surface topography [4]. Surface characteristics like topography, chemistry or surface energy play an essential role in osseointegration, therefore surface quality can have an effect on this process. It was the goal of this study to investigate the biocompatibility and equivalency of the treated surface in a cell- and rabbit model, with the clinically established untreated surface serving as a reference.

Material and Methods

Two kinds of samples were made from three titanium alloys (cpTi, Ti-6Al-4V and Ti-6Al-7Nb). All samples were alumina-blasted with 3 or 4 bars depending on the alloy to reach a Ra of 4-6 µm. Half of these samples were cleaned with the new method and all samples were gamma sterilized.

For the cell study human bone cells (HBC) were freshly isolated from three different donors. After cultivation the cells were plated on discs. For two days the cells were kept under proliferation medium and then were switched to differentiation medium. The cells were cultured for 6 to 12 days. Distribution and proliferation was determined by DiI staining during the cultivation. Differentiation capacity was tested by antibody staining for bone specific ALP (bALP). RNA was isolated and analysed by PCR for Osteocalcin, Collagen I and 18S RNA. Attachment and spreading was examined by scanning electron microscopy and by staining of the cytoskeleton with anti-Actin and anti-Vinculin antibodies.

For the animal study cylinders were implanted into the femoral condyle of rabbits for 4 and 8 weeks. Thereafter bone histologies were made with a bone specific staining. The probes were analysed for adverse tissue reaction and bony ongrowth.

Results and Discussion

The plating efficiency was the same on both materials. During the whole cultivation time no depletion of cells or increase in dead cells in the medium were seen.

A difference in the cell distribution could be observed depending on the cultivation time. In the control group the cells showed an even distribution on the material during the complete culturing time. On the treated samples a migration of the cells did occur after 5 days and the cells started to form colonies on the material. These colonies still showed proliferation and cells showed a normal phenotype on the material. The colony forming may represent the first step of bone nodule formation although it starts very early in this case. To further investigate this we have to examine if the colonies are calcified. On the other hand the colony forming may also depend on surface energy distribution that could lead to preferred adhesion of the cells.

After 4 days in differentiation medium only a very low amount of bALP could be detected, but after 6 days in differentiation medium the expression of bALP was very high on all samples compared to a non stimulated control.

The actin staining showed a normal cytoskeleton on all samples. No specific staining of vinculin could be identified, due to a strong fluorescence reflection by the metal surface.

With quantitative PCR, osteocalcin, bALP and collagen I could be detected in all samples in the same range after 10 days in culture. Also in all of the three tested HBC lines the amounts were in the same range.

In the animal study both surfaces showed a beginning bone repair after 4 weeks and no inflammatory reaction could be seen. After 8 weeks the bony ingrowth was completed and no difference could be detected between the controls and the treated surfaces.

Conclusion

- We are able to produce a new alumina particles free surface without changes to the surface morphology.
- The new surface treatment does not alter the biocompatibility of the implant surface.
- In vivo the new treated surface shows equivalent bone ongrowth as the established grit blasted surface.

Acknowledgements

We would like to thank the EMPA St. Gallen for the cell study and the University Hospital Aachen for performing the animal study and the production of the histologies.

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BINDING COLLAGEN (I) FRAGMENTS TO TITANIUM OXIDE SURFACES: A MOLECULAR DYNAMICS STUDY

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Introduction

The adsorption of proteins of the extracellular matrix on titanium oxide is the first step of tissue growth on titanium implants covered by a passivation layer. Collagens are very important cell adhesion proteins. Here we present molecular dynamics simulations of collagen I fragments on (100) rutile.

A characteristic feature of the protein adsorption on many metal oxides is that it occurs in two steps, the first one leading to a weak adhesion whereas on a longer time scale strongly bound molecules are observed which spread out and cover a large surface area. The slower second process is considered to introduce conformational changes into the molecule, which may be reversible or irreversible [1].

Method of Calculation

Triple helices with lengths of about 9 nm were assembled from two strands of 30 residues with α_1 sequence and an α_2 one with 29 residues. The latter one contained the peptide sequence RGD (Arginine-Glycine-Aspartic acid) which is of fundamental importance for cell adhesion. The titanium oxide surface was modelled as a rigid slab with perfect geometry or surface terraces. The surface protonation and hydroxylation equilibria were adjusted according to a pH-value of 7.4 [2], the resulting net charge being balanced by Na^+ -counterions. The protein molecule was embedded in water molecules in a periodic cell of about $14 \times 3 \times 6 \text{ nm}^3$ in size. Molecular dynamics runs employed the AMBER7 force field scanning at least 1600 ps for each configuration.

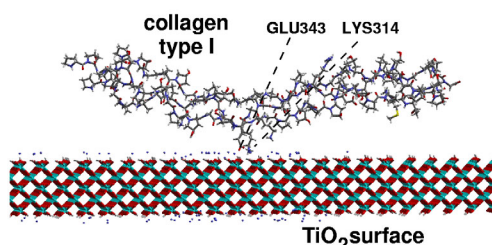


Fig. 1 Collagen fragment on a perfect (100) rutile surface

The unconstrained triple helix shows some untwisting at the ends during this time, inducing opening of the hydrogen bonds between the strands in the final two residues. We constrained the final hydrogen bonds in most runs mimicking a periodic system.

Results and Discussion

The protein attaches to the surface only at few contact points employing residues with charged side chains such as glutamic acid and lysine. The NH_3^+ group of lysine shows torsional motion and opening and closing of hydrogen bonds as was found in earlier Car-Parrinello simulations [3]. In spite of this dynamics the group appears to be immobilized on the surface. Between glutamic acid and the surface the formation of stable hydrogen bonds was observed. The conformation of the triple helix as revealed by Ramachandran plots is not significantly influenced by the interaction of the protein with the surface, but the fragments bended significantly during the run time, which was not observed in solutions without TiO_2 and is attributed to the interaction with the charged surface.

Conclusions

The adsorption process found here results in a weak bonding of the molecule to the surface by only a few contact points, irrespective of the surface roughness. This has to be assigned to the fast process. The atomistic simulation, however, gives some idea on the nature of the more important slow process. The number of contact points tends to increase during the simulation, which may lead to deformation and spreading of the molecule. The hydrogen bonds are likely to break under the influence of strong external forces. In addition to that covalent bonding of the protein to the surface is possible by ester condensation between surface hydroxyls and protein carboxyl groups [3], which may contribute to the strong bonding attained during the slow process.

Acknowledgements

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CRITICAL REFLECTION OF THE STATISTICAL CORRELATION BETWEEN BIOMATERIAL AND CELLULAR PARAMETERS

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Introduction

For mathematical modelling of the biomaterial-cell contact it is necessary to find both parameters characterizing physical and chemical properties of the material surface and also such describing the reaction of the adhering cells. Only those material and cell parameters that correlate with each other are applicable to model this contact mathematically. Only few papers are dealing with this special problem [1, 2].

The aim of this paper is to present results of physical/chemical and biological investigations made on differently modified rough titanium implant surfaces in order to find out only the correlating parameters. Furthermore we want to discuss several ways to apply statistical methods to the correlation problem.

Experimental

The surface structure of cp-titanium samples was modified in a range of roughness average R_a from 0.07 μm to 7 μm by several modification methods (polishing, machining, etching and blasting with glass balls/corundum particles). For the physical characterization of the surface morphology both standardized roughness parameters (ISO 4287) and additional parameters like fractal dimension D_f and topothesy K were calculated from the surface profile [3]. Additional electrochemical parameters were determined by methods of Linear Sweep Voltammetry, Chronoamperometry and Electrochemical Impedance Spectroscopy.

Cellular investigations were carried out with MG-63 osteoblastic cells. Cells were cultured in DMEM with 10% fetal calf serum (FCS) and 1% gentamycin (Ratiopharm GmbH, Ulm, Germany) at 37°C and in a 5% CO_2 atmosphere. In general, cells were seeded with a density of 3×10^4 cells/ cm^2 onto the titanium materials and into control dishes. Following cellular parameters were investigated to evaluate the correlation to physical/chemical properties of the titanium: Adhesion, spreading, integrin expression, and length of integrin contacts.

Correlation between material and biological parameters was made by means of the statistical program SPSS presuming a linear dependence. Because of the specific measurements of material and cellular parameters we couldn't build pairs of variates from single measurements. That's why we had to average the data. This was done in different ways.

Results and Discussion

Only few ones of all investigated parameters both on

material and on cellular side were applicable for correlation. For example we found in our studies that fractal structure parameter topothesy has influence on the spreading behaviour of the osteoblastic cells (fig.1). However the correlation coefficient and its statistical significance heavily depend on the method of averaging the available data. Especially the biological data (spreading area) were afflicted with relatively high error up to 30%. Averaging of this data masks the true facts. That's why the correlation coefficient considerably decreases if the biological parameters are not averaged. On the other hand the statistical reliability increases due to the higher number of investigated cases.

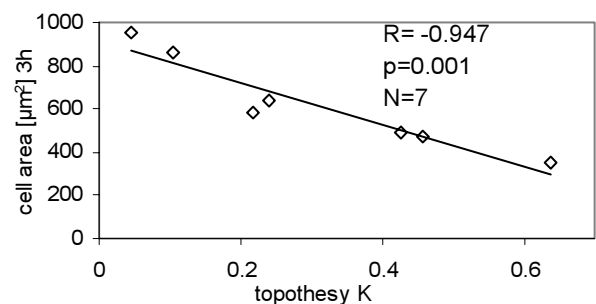


Figure 1: Linear dependence between material parameter topothesy K and cell spreading area (3h). The $N=7$ pairs of variates were got by averaging both material and cell parameters for each material modification.

Conclusions

Critical error discussion is necessary in statistical correlation between material and biological parameters. Often the results are heavily influenced by the statistical handling of data, especially if only few data are available. May be that new unconventional methods like bootstrap method can show a way out of this dilemma [1].

Acknowledgement

We thank the DFG for financially supporting this work in the frame of SPP 1100 and also DOT Ltd. for providing the titanium samples.

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INFLUENCE OF THE ZINC CONCENTRATION OF SOL-GEL DERIVED ZINC SUBSTITUTED HYDROXYAPATITE ON CYTOKINE PRODUCTION BY HUMAN MONOCYTES *IN VITRO*

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Introduction

A possible complication associated with the implantation of hydroxyapatite (HA)-based prosthesis is the release of particles. These particles can be phagocytosed by monocytes that are among the first cells to colonize the inflammatory site.

The activated monocytes produce inflammatory mediators such as cytokines that cause osteoclasts activation [1]. The present work, describes studies on the effect of sol-gel derived zinc-substituted HA particles with various zinc substitution percentages (0.5 – 2%) on cytokine production (TNF- α , IL-1 β , IL-6, IL-10, and IL-8) by both LPS-stimulated or unstimulated human monocytes.

Materials and methods

THP-1 human monocytes (American Type Culture Collection, Manassas, VA, USA) were used to evaluate cytokines synthesis. THP-1 cells cultured alone was the control. THP-1 cells were exposed to LPS (1 $\mu\text{g}\cdot\text{ml}^{-1}$) as a positive control and to evaluate the effect of zinc-substituted HA on stimulated cells. In the present work, the surface area ratio (SAR = surface area of material/surface area of cell) method was used. Kruskal & Wallis non-parametric test was performed ($p < 0.05$).

Results

Our data demonstrates that the zinc has an effect on cytokines production [2]. It decreases the production of TNF- α (fig 1) and increases the production of IL-8 by unstimulated cells. Using LPS-stimulated cells, it decreases the production of inflammatory cytokines and increases the production of anti-inflammatory cytokine (IL-10), indicating that zinc-substituted hydroxyapatite has favourable effects on the cytokines production by monocytes.

Conclusions

Zinc-substituted hydroxyapatite had an effect on the production of cytokines by human monocytes cells. The

production of TNF- α by unstimulated cells decreased with the zinc concentration. Additionally, unstimulated cells demonstrated an increase in the production of IL-8, indicating that increasing zinc concentration has an enhancing effect on chemotaxis. Using LPS-stimulated cells, the production of IL-1 β and IL-6 decreased when zinc-substituted HA powders were used indicating that the inflammatory reaction is decreased compared to pure hydroxyapatite powders. The production of IL-10 by stimulated cells increased with the concentration of zinc. So zinc has positive effects: it decreases the inflammatory reaction, increases the chemotaxis and down-regulates the inflammatory reaction.

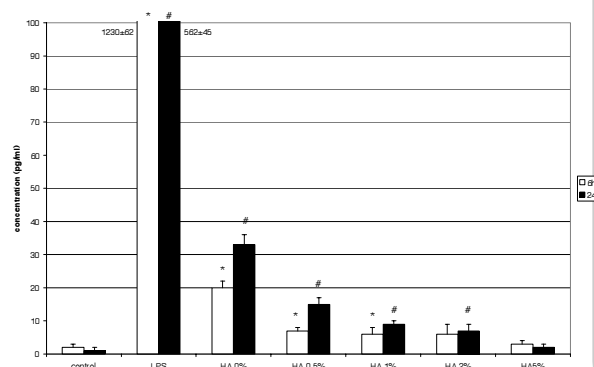


Figure 1: TNF- α production by monocytes exposed to THP-1.

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CYTOKINE RELEASE OF HUMAN OSTEOBLASTS EXPOSED TO WEAR PARTICLES GENERATED AT THE INTERFACE OF THE FEMORAL STEM AND BONE CEMENT

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Introduction

The main reason for early failure of total hip replacement is aseptic loosening [1], which is seen to be as an inflammatory and osteolytic reaction caused by particulate wear debris [2]. Abrasive wear is especially a consequence of micro-motions between the components of modular built endoprostheses and at the interface of implant and bone cement. Direct impact of wear products on osteoblasts function and bone remodelling induced by bone resorptive cytokines was described.

The purpose of this study was to examine the variety of cytokines released by human osteoblasts exposed to different abrasive wear particles, which were generated in an in-vitro test model representing the interfacial wear between total hip stem and the surrounding bone cement.

Material and methods

The wear particles were produced by oscillating definite micro-motions between femoral stem and the bone cement in combination with a constant radial contact force ($\pm 250\mu\text{m}$; 3×10^6 cycles; 5Hz; 0,64kN). For the following cellular investigations we employed particles generated from identically designed hip stems with two different material compositions (Ti-6Al-7Nb and Co-28Cr-6Mo) and two surface topographies (rough and smooth) combined with standard PMMA bone cement containing zirconium oxide as radio opacifier. We compare them with commercially available pure titanium particles (cp-Ti) and particulate zirconium oxide (ZrO₂). For analysing the inflammatory response, human osteoblasts cultured from bone marrow were exposed to particles of all different entities at concentrations of 0.01, 0.1 and 1.0 mg/ml. After challenge the releases of different cytokines, e.g. interleukin-6 (IL-6), interleukin-8 (IL-8) and tumor necrosis factor alpha (TNF- α), were determined by the use of a Multiplex Cytokine Assay (Bio-Rad Laboratories, München) capable to detect 5ng/ml of the each protein.

Results and Discussion

The results revealed distinct effects on the cytokine release of human osteoblasts towards particulate debris in a dose dependent manner. At particle concentrations of 1.0 mg/ml there was a trend of severe inhibition in the release of IL-6 and IL-8 of about ten times in comparison to the particle-free control for all material compositions. Except zirconium oxide particles which deviated not significantly. On the other hand in some cases lower concentrations stimulated the release of interleukin-6 with reference to the particle-free value, i.e. cp-Ti and the abrasive wear of the rough Ti-6Al-7Nb-

stem. However, differences could be observed in the arrangement of the individual particle entities to each other, but there is no statistical significance. Thereby, particles from prostheses with rough surface finishes as well as cp-Ti caused greater inhibition in cytokine release at high concentrations, probably due to massive cell damage. Otherwise lower concentrations induced cytokine releases and maintained thus the inflammatory reaction favouring osteolysis. Latter one was similar to particulate zirconium oxide, which expressed high amounts of cytokines at each concentration level.

With regard to the other examined cytokines, IL-2, IL-4, IL-10 and GM-CSF could not be verified neither after an incubation period of 4 nor 9 days. The expression of TNF- α were demonstrated after 4 days, whereas IFN- γ had its peak after 9 days, but in each case the values ranged about the limit of detection and showed no statistical differences.

In summary, there was a trend towards greater influence of the cytokine release with rough than with smooth surfaces displaying a wider range of the amount of released pro-osteolytic mediators. This, combined with our previous studies and the fact that cp-Ti had closely the same effects, suggests that especially the metallic wear affects growth and metabolism of osteoblasts ending in aseptic loosening. However particulate zirconium oxide does not directly damage osteoblasts, it supports probably at least the inflammatory reaction.

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POLYMER BASED GENE DELIVERY FOR ARTIFICIAL HEART VALVE

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Objective

Artificial heart valve application is mainly limited to the high risk of acute thrombosis and chronic instability of the implant surface. Direct seeding of endothelial cells on the heart valve surface suffers from a low viability of the cells and the high risk of thrombosis. To overcome the above problems, a decellularized biological scaffold with mechanism of controlled release therapeutic DNA from the scaffold was developed to regulate cellular attachment and tissue regeneration over the scaffold.

Materials and Methods

In this study, the porcine aortic root was decellularized by enzymatic removal of the endothelial cell layer and cellular components [1]. PEI/DNA complexes were immobilized into the decellularized scaffold by fibronectin. Marker genes Laz and GFP were employed to evaluate the gene expression in the rat coronary endothelial (RCE) cells seeded on the scaffold by immunohistology staining.

Results and Discussion

Decellularization: The decellularization process removed all cellular components of the porcine aortic wall. After enzymatic removal of the endothelial cell layer and cellular components of the media, the fibrous network of collagen and elastin, and proteoglycans forms the surface of the scaffold as shown in Figure 1. H&E stain of the aortic media after enzymatic decellularization demonstrated complete removal of all cellular material, and it showed that the decellularization process kept the surface morphology of the decellularized matrix, preserving the tissue native texture and microporosity, thus facilitating adhesion and migration of recipient cells.

Plasmid DNA incorporation, release, and in vitro transfection: The transfection efficacy of PEI/DNA complexes released from decellularized matrix was assessed using GFP as reporter genes. The complexes of PEI/DNA in the N/P ratio of 17 [2] were incorporated with decellularized matrix by fibronectin. The transfection of GFP plasmid released from decellularized matrix was assessed by transfecting rat coronary endothelial (RCE) cells. Immunohistology staining shown in Figure 2 demonstrated that there is a confluent layer of endothelial cells covered the mural surface of the decellularized matrix and the endothelial cells seeded

on the matrix got transfected by the GFP plasmid released from the matrix, suggesting that the incorporated and released DNA remained functional.

Conclusion

The decellularized biological xenogenic scaffold integrated with mechanism of controlled and sustained release of plasmid DNA may open up new prospects to explore the functional effects of the expression of desired genes for cellular attachment and tissue regeneration in tissue engineering.

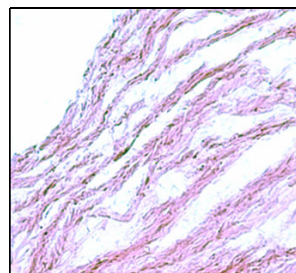


Figure 1: Decellularization of porcine aortic wall

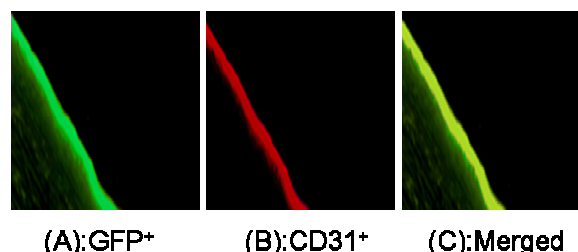


Figure 2: Immunohistology staining of a xenogenic decellularized biomatrix

Acknowledgements

This work was supported by Miltenyi Biotec (Germany), Steinbeis TransferZentrum fuer Herz-Kreislaufforschung (Rostock), and German Helmholtz Foundation University Research Group.

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TISSUE ENGINEERING WITH POLYESTERAMIDE DERIVED NONWOVENS

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The reconstruction of soft tissue defects remains still a problem in plastic and reconstructive surgery. Adipose tissue engineering offers new perspectives since there are limited surgical possibilities. Preadipocytes, adipogenic precursor cells, which are located between mature adipocytes in adipose tissue, represent a powerful tool for soft tissue engineering due to their ability to proliferate and differentiate to mature adipose tissue [1]. This study presents a novel material made of a bioabsorbable copolyesteramide. Polymers are established biomaterials due to their chemical characteristics and their flexibility for material processing and modifications [2]. An appropriate structure of the scaffold surface is required not to inhibit the growth or differentiation since maturation leads to augmentation of the cells by storing lipid vacuoles. In this study, nonwoven structures were generated by textile manufacturing using an aerodynamic web formation process and a needle felting technique. Scaffolds were then seeded with preadipocytes and evaluated for allowing preadipocyte proliferation and differentiation. Our findings demonstrate the applicability of polyesteramides for tissue engineering and are a positive guidance towards an optimally prepared scaffold for in vivo use.

Materials and Methods

For seeding of nonwovens, surface contaminations were removed by a surface cleaning procedure followed by vacuum drying and gamma-sterilisation. Preadipocytes were isolated out of human subcutaneous adipose tissue of healthy patients who had undergone elective operations. After the isolation procedure, preadipocytes were cultivated in culture dishes until confluency, then trypsinised and seeded on the scaffolds. Nonwoven carriers were either precoated with fibronectin or directly used for seeding. Scaffolds were seeded and then examined for allowing proliferation and differentiation.

Results

The analyses show good adherence and spreading of preadipocytes on polyesteramide derived nonwovens. Cells are adequately distributed and most cells are attached to fibers. To determine preadipocyte viability on scaffolds, formazan formation was analysed 6h and 9d after seeding preadipocytes on fibronectin-coated versus

uncoated nonwovens (see Tbl. 1). Precoating with fibronectin displays a significantly higher cell number. After 9 days there was still a significant difference but not as strong as on day 1. Fibronectin coated nonwovens also allow good differentiation, especially in areas where cells appear in clusters.

Table I: Growth of preadipocytes on fibronectin-coated versus uncoated nonwovens

		number of cells	
		6h post seeding	9d post seeding
number of cells	no fibronectin	100%	252,66%±31,67%
	with fibronectin	242,9%±97,76%	328,91%±46,23%

Discussion and Conclusion

The results of this study show that polyesteramides are promising materials for tissue engineering, especially due to the pH-stability during degradation. This prevents the formation of acid degradation products which are often responsible for inflammatory reactions after scaffold implantations. Furthermore, fibronectin precoating shows beneficial effects on cell adherence to nonwovens since it elevates the amount of attached cells and enhances conversion to mature fat cells. These findings have high potential for clinical purposes and are encouraging for further in vivo experiments.

Acknowledgements

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AGGREGATION AND CO-PRECIIPITATION OF POLYSIALIC ACID

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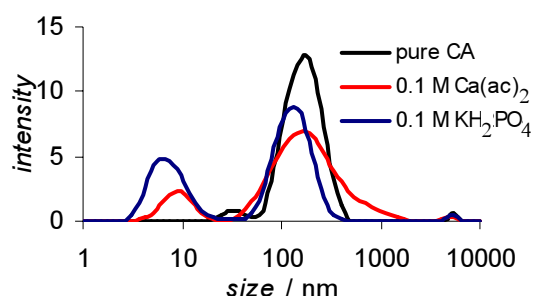
Introduction

Optimized bone replacement materials can be degraded by the body and enable the rebuilding of bone. Often, composite materials of an organic polymer and inorganic calcium phosphate are employed as resorbable implant materials. However, the degradation of the organic polymer often leads to degradation products which are detrimental to an effective healing process (e.g. polylactic acid), and usually this process occurs in an uncontrolled manner, for example when polysaccharides as hyaluronic acid or chitosan are used. Polysialic acid is a special endogenous polysaccharide, which takes part in the formation of the nervous system during the embryonic stage. In the adult body, the polysialic acid-degrading enzyme is inactive. However, degrading enzymes can be administered, and therefore the degradation process can be controlled. This property and The optimal biocompatibility and the controlled degradation propose a high potential for polysialic acid as a biomaterial.

Materials and Methodes

Dynamic light scattering (DLS) enables the measurement of the hydrodynamic radius of particles in solution (Instrument: Malvern instrument, Zetasizer Nano-ZS). For the measurements, colominic acid (CA), the sodium salt of polysialic acid was used (nacalai tesque). To investigate the influence of the addition of different salts on the aggregation of CA, three different solutions were compared. 0.05 g CA was dissolved in bidist. water, in 0.1 M KH_2PO_4 or in 0.1 M $\text{Ca}(\text{CH}_3\text{COO})_2$. These two salts were also used in co-precipitation reactions, which were conducted in order to obtain polysialic acid-calciumphosphate composites.

Figure 1: DLS measurements of CA with and without salt addition



Results and Discussion

The DLS measurements show that the addition of salts has a strong influence aggregation of CA. In distilled water, aggregates with a mean diameter of 160 nm are present. To a large part, these particles de-aggregate upon the addition of salt to form individual molecules, represented by a peak at 9 nm (Figure 1). This process can be used to produce nanostructured composite materials with calcium phosphate (Figure 2). The composite shows a homogeneous fine structure down to the nanometer level and in this respect resemble the structure of natural bone.

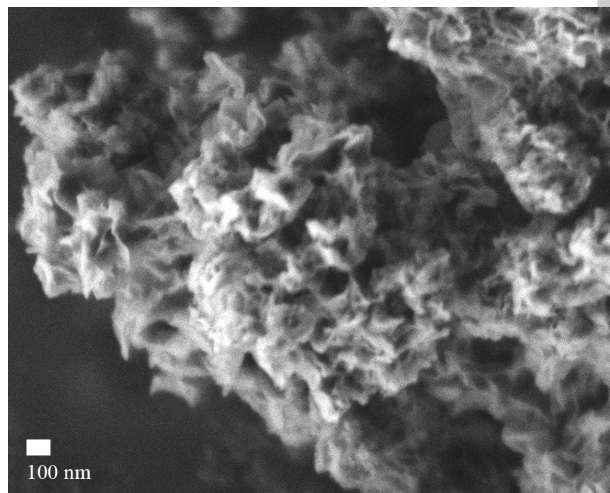


Figure 2: SEM image of a polysialic acid-calcium phosphate composite.

Conclusion

In aqueous solution, pure polysialic acid forms large aggregates. The addition of salts leads to a deaggregation. This effect can be used to generate nanostructured polysialic acid-hydroxyl apatite composites.

Outlook

The novel composite materials will be tested in cell cultures to test their biocompatibility and bioactivity. Furthermore, it will be investigated whether the material can be degraded by the polysialic acid-degrading enzyme endosialidase (EndoN).

Acknowledgements

We would like to thank the DFG for the financial support of this project within the framework of the research group FOR 548 "Polysialic acid".

CHANGES OF THE SURFACES OF DIFFERENT MAGNESIUM ALLOYS AS DEGRADABLE IMPLANTS DURING DEGRADATION IN RABBIT TIBIAE

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Introduction

Magnesium alloys (MA) as degradable metal implants provide a higher E-modulus and tensile yield strength than the widely used polymeric implants [1]. Therefore they are presumably useful for fracture repair in weight-bearing bones. However, medical use of MA as implants is limited due to the fast corrosion rate and gas production during degradation [2]. Earlier investigations demonstrated reduction of the corrosion rate by addition of rare earth elements, but machined pins still showed irregular corrosion and gas production [3]. Due to the better homogeneity of extruded pins, it was the aim of this study to investigate the changes of the surfaces during degradation of three different extruded MA in rabbit tibia.

Materials

Extruded pins (2,5 mm x 2,5 cm) of LAE442, WE43 and MgCa0,8%, 20 of each MA, were implanted intramedullary into both tibiae of ten rabbits. Radiographs were taken directly postoperative and then once a week. After three and six months after surgery five of the animals of each group were euthanized and both tibiae were explanted for further analysis. Micro-computed tomography (μ CT) of the right tibiae, and reflected light microscopy as well as scanning electron microscopy (SEM) and energy-dispersive X-ray (EDX) of the explanted pins were performed.

Results & Discussion

Until the end of the investigation period, the implants showed no changes in diameter or length radiographically. A decrease in density of the pins occurred at different times, but no gas production was seen. With μ -CT new endosteal bone formation could be seen close to the implants. Light microscopy and SEM revealed corrosion on the surfaces combined with cell adhesions on all implants. MgCa(0,8) shows the most distinct changes of the implant surface after six months, while the surfaces of LAE442 implants showed no changes except of some small clefts. On the surfaces of the WE43 implants, an irregular delamination of the surfaces could be seen and in addition some bigger clefts in comparison to LAE442 (Fig. 1). The EDX analysis of the implanted pins showed an accumulation of calcium and phosphorus which may have originated from surrounding bone.

Conclusion

All extruded pins of the three MA showed a relatively slow and uniform degradation rate. The cell adhesions

on the implant surfaces demonstrated a good biocompatibility of the MA. The rare earth containing implant LAE442 showed only minor changes and MgCa(0,8) the most distinct changes of the surfaces. Further investigations are necessary to show the effects of the different changes of the surfaces on the stability of the implants.

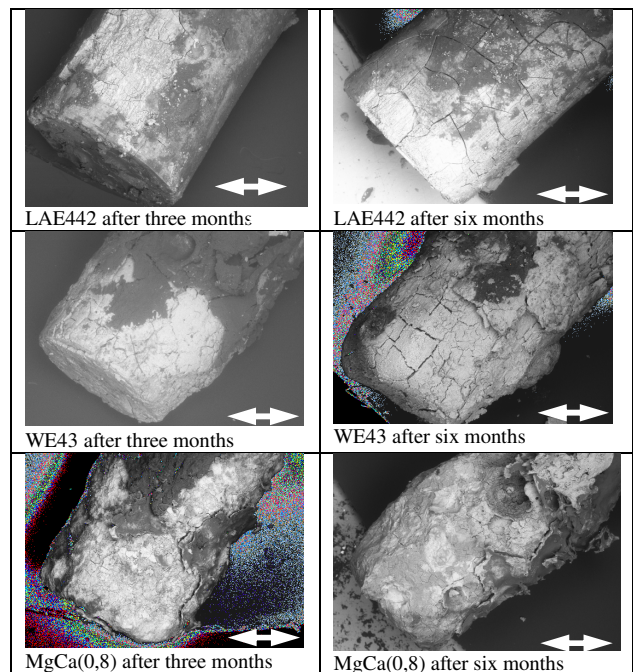


Figure 1: SEM of the explanted pins three and six months after implantation (= 1mm)

Acknowledgements

This study is part of the collaborative research centre 599, which is sponsored by the DFG.

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ENHANCED BIOCOMPATIBILITY OF HUMAN ENDOTHELIAL CELLS ON MODIFIED POLYURETHANE SURFACES

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Introduction

To improve the biocompatibility of polyurethane (PUR) in blood-contacted devices, we modified PUR surfaces by ion irradiation or plasma vapor deposition (PVD). Adhesion, proliferation, and cytotoxicity of human umbilical vein endothelial cells (HUVEC) grown on engineered surfaces were compared to unmodified controls (uPUR). Surface degradation was also investigated.

Materials and Methods

Ion irradiation was done using C, O, N, or Ar ions with energies of 0.3 – 50 keV and doses of 10^{13} – 10^{15} ions/cm². Zircon or titanium were used for PVD-coatings. A triple immunostaining was achieved for the adhesion studies. The percentage of apoptotic cells was calculated after DAPI staining. Cell proliferation was analyzed using WST-1 assay. PUR surface degradation by HUVEC was investigated after 6 weeks. Cells were removed before scanning electron microscopy (SEM) analysis. Surface topography and free energy were checked by atomic force microscopy and contact angle measurement, respectively.

Results and Discussion

HUVECs showed an increased adhesion and proliferation on ion implanted or PVD-coated surfaces compared to uPUR (Fig. 1). Intense spreading and most focal contacts were observed on N-PUR or PVD-coated PUR in contrast to uPUR and C-PUR. Cell proliferation was higher and apoptosis rate (Tab. 1) was lower on all modified surfaces. Ion energy had a stronger effect on cell counts (24 h) and cell proliferation (5 d) than ion dose. PUR surfaces bombarded with high ion energies and doses showed higher roughness and lower water-contact angles (i.e. hydrophobic) and were therefore less favorable for cell adhesion. A time-dependent degradation caused by HUVECs was observed by SEM. PUR surfaces with cell contact for 1 week were morphologically intact, while those with 6 week contact displayed various defects e.g. rafts and swellings (Fig. 2). Among all, the PVD-coated PUR surfaces seem to be most stable against enzymatic degradation.

Conclusions

Ion implantation or PVD-coating of PUR surfaces significantly improved adhesion and proliferation of endothelial cells. These modifications also significantly reduced apoptosis rates. However, in most cases the stability of PUR to surface degradation was not significantly enhanced. For Zr- or Ti-coated PUR, stability might be improved by achieving some additional surface changes e.g. thickening of the coat layer. Therefore, surface-engineered PUR may be a promising

material in cardiovascular applications such as blood vessel implants.

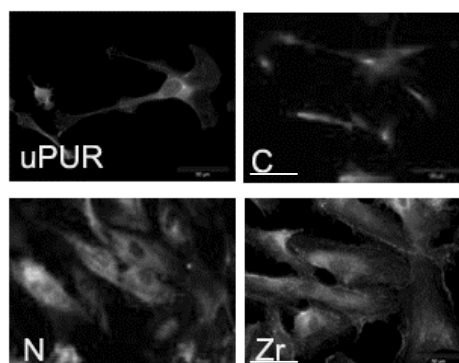


Figure 1: Improved HUVEC adhesion (24 h) on ion-implanted (C, N, 10^{13} ions/cm², 0.3 keV) or PVD-coated (Zr, 50nm) PUR surfaces in comparison to uPUR. Staining: phalloidin (actin cytoskeleton), vinculin (focal contacts) and DAPI (cell nuclei). Bars: 50µm.

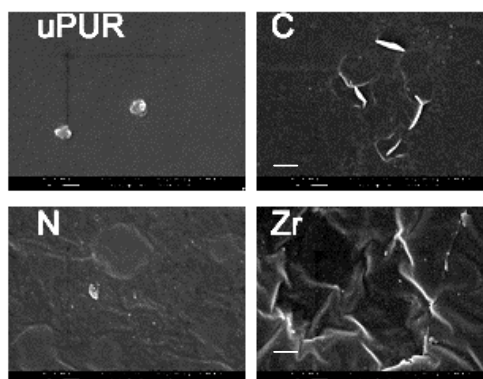


Figure 2: Surface defects of PUR caused by HUVECs after 6 weeks of cell contact. Cells were removed by a short trypsination step before SEM analysis. Bars: 1µm.

Table 1: % Apoptosis of HUVECs grown on PUR surfaces (24 h). Mean values of 3 microscopic visual fields are given.

uPUR	N-PUR	C-PUR	Zr.PUR
33.3	2.9	7.8	3.2

Acknowledgements

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MINERALIZED CELLULOSE SCAFFOLDS FOR CARTILAGE TISSUE ENGINEERING

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Introduction

An important field for the application of tissue-engineered cartilage are osteochondral defects in joints. Treatment of these defects with tissue-engineered cartilage still involves the problem of fixation and integration into the surrounding tissue. Ideally, the design of a tissue engineered osteochondral graft must address not only the damaged cartilage but also the subchondral bone to allow for adequate osteochondral repair and to integrate the neo-cartilage into the osseous surrounding. In this study we demonstrate the chondrocyte cell response on calcium phosphate coated cellulose fabrics.

Materials

Non-woven cellulose-II fabrics with a specific mass of 257 g/m² (Lyocell®, Lenzing, Austria) were used as scaffolds in this study. The samples were suspended in a saturated Ca(OH)₂ suspension and subsequently exposed to 1.5 SBF (simulated body fluid) for 2 weeks in order to coat the cellulose scaffolds biomimetically with calcium phosphate [1,2]. The scaffolds were sterilized by water steam sterilization and seeded with primary bovine chondrocytes. Cell vitality was quantified and histological analyses of proteoglycan synthesis as well as immunohistology of collagen type I and II were carried out.

Results and Discussion

A surface layer consisting of spherical aggregates covered the Ca(OH)₂ pre-treated cellulose fibers after soaking in 1.5 SBF for 2 weeks. The nano-crystalline precipitates were composed of hydroxy carbonated apatite (HCA, $\text{Ca}_{10-x}(\text{HPO}_4)_x(\text{CO}_3)_y(\text{PO}_4)_{6-x}(\text{OH})_{2-x}$, with $0 < y < x < 1$) with a composition similar to the inorganic part of bone, brushite ($\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$) and calcite (CaCO_3). All these materials are referred to as biocompatible and degradable in physiological environment. Scaffolds could be sterilized without changing the composition.

The scaffolds were seeded with a chondrocyte solution (5×10^6 cells). After one week in culture the fiber surface was covered with vital chondrocytes (98.9 % vitality). Cell density was high and cells were spread (Figure 1), indicating the biocompatibility of these materials. Cartilage development was investigated using primary bovine cartilage cells cultured for 6 weeks. The HCA-coated scaffolds were completely covered with cartilaginous tissue. The cells were vital and matrix

expression was observable. Collagen II was detected in the bulk of the scaffolds, whereas in the interface between scaffold surface and physiological fluid mainly collagen I was detected. This might be explained by differences in the supply with nutritive factors inside the scaffold and on its surface affecting the protein expression behaviour of adhered cells.

In contact with a physiological chondrocyte solution, calcium is expected to be leached out from the precipitated layer, which might lead to a microenvironment that triggers the development of cartilage in a way similar to cartilage repair in the vicinity of subchondral bone.

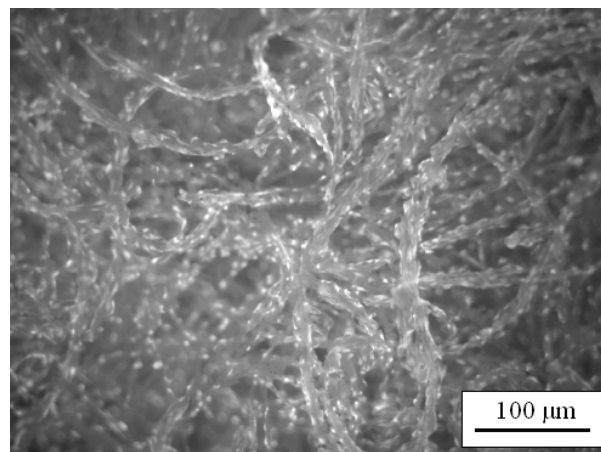


Figure 1: HCA coated cellulose scaffold seeded with primary chondrocytes.

Conclusions

Porous cellulose structures activated in Ca(OH)₂ solution and subsequently coated with a calcium phosphate layer precipitated from a supersaturated physiological solution are considered as novel scaffold architectures for *in vitro* cartilage tissue engineering. A homogeneous distribution of seeded chondrocytes is possible. The development of cartilaginous tissue could be proved.

Acknowledgements

DFG is thankfully acknowledged for financial support.

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ESTABLISHMENT OF AN IMMORTALIZED ADULT HUMAN MESENCHYMAL STEM CELL LINE TO STUDY OSTEOGENIC DIFFERENTIATION

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Introduction

Human mesenchymal stem cells (hMSCs) are present in various tissues including bone marrow and can be isolated to use them in experimental approaches. However, there is a considerable variation in number and full differentiation potential which is donor dependent. Therefore, the establishment of a permanent cell line with characteristics of a mesenchymal stem cell to use as a model for experimental studies could be advantageous. We established a hMSC cell line that is suitable to study the biology of these cells in the interaction with biomaterial surfaces.

Materials and Methods

Mesenchymal stem cells from human bone marrow were isolated according a standard procedure which includes separation by density gradient centrifugation [1] and adhesion of the cells on plastics. Adherent cells characterized as mesenchymal stem cells were cultured for 14 days in DMEM with 10% FCS and 1% antibiotics/antimycotics.

To immortalize cells, the culture was infected with an adenovirus 12-SV40 virus (Ad12-SV40) [2]. Efficacy of immortalization was tested by staining for SV40 large T. This cell line we named KM57Ad. The differentiation potential of the primary cells isolated from bone marrow and of the immortalized cells to generate both osteoblasts and adipocytes was verified. Osteogenic differentiation was analyzed by quantitative real time RT-PCR. Expression of alkaline phosphatase (ALP) was also tested by fast red staining. Oil red O staining was used to visualize the adipogenic differentiation.

Results and Discussion

First SV40 large T antigen positive cells were detected 6 days after infection. Population doubling was within 2 days. The KM57Ad cells maintained a spindle-like phenotype, similar to the unimmortalized cells, up to 18 passages. They show a clonogenic growth typical for

stem cells. The potential to differentiate into both osteoblasts and adipocytes was positively tested for the primary cells as cell source for the cell line and also for KM57Ad cells which indicates that infection with the adenovirus did not change the characteristics of a mesenchymal stem cell. After a 14 days culture in osteogenic differentiation medium both cell types expressed osteogenic differentiation markers, like ALP, collagen, osteocalcin and bone sialoprotein.

After 21 days in a culture containing adipogenic medium adipocyte differentiation in both cell types were visualized by Oil red O staining.

Conclusion

Immortalization of hMSC by Ad12-SV40 was successful to establish a cell line with characteristics of a mesenchymal stem cell. The cells maintained the stem cell phenotype, they were able for self-renewing and to differentiate into multiple mesoderm-type cell lineages. Thus, the cell line KM57 Ad could be a useful tool to study cell differentiation in vitro using tissue engineering protocols.

Acknowledgements

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We thank Annelie Peters for excellent technical assistance.

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FIBRONECTIN ENHANCES CELL ADHESION ON A HYDROPHOBIC FLUOROCARBON MODEL SURFACE

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Introduction

Anchorage-dependent cells like osteoblasts or fibroblasts attach to material surfaces through binding to specific adhesion proteins or by unspecific interaction with the material surface. Cell adhesion to a surface is supported by receptors like integrins in the cell membrane associated with the cytoskeleton. Proteins adsorbed from blood serum or interstitial fluids influence attachment of cells to biomaterial surfaces. Here, we analyzed the adhesion of human MG63 osteoblasts and 3T3 mouse fibroblasts to self-assembled monolayers (SAMs) pre-coated with serum proteins or human fibronectin.

Materials and Methods

Silicon wafers were oxidized in nitric acid solution to create hydrophilic surfaces (Ox). Further modification of the wafer surfaces was achieved by coating with self-assembled monolayers (SAMs) exhibiting poly(ethylene glycol) (PEG), hydrocarbon (OTS), or fluorocarbon (HFS) moieties. Chemically modified surfaces were characterized by XPS and measurement of the advancing water contact angles (θ_a) using the sessile drop method. Wafers were pre-coated with fetal bovine serum or with human fibronectin (FN, 5 μ g/ml in PBS) for 1 hour at 37°C. Adsorbed proteins were quantified directly on the wafers by chemiluminescence. Changes in surface wettability were measured by water contact angle. Human MG-63 osteoblasts and mouse 3T3 fibroblasts were seeded in MEME supplemented with 10% FBS onto sterilized coated and uncoated wafer specimens. The cells (1×10^5 cells/well) were then incubated for 5h, and the number of cells on each surface was determined using a crystal violet assay. Tissue culture polystyrene (PS) served as a reference material. Cell morphology and spreading on wafers was also characterized by SEM.

Results and Discussion

Adsorption of serum proteins was high on oxidized (Ox) and lowest on fluorocarbon-modified (HFS) surfaces. Lowest amounts of adsorbed fibronectin were found on PEG- and HFS-coated wafers. Hydrophobic surfaces became more hydrophilic due to protein pre-coating. The highest number of MG63 osteoblasts was detected on hydrophilic oxidized surfaces (Ox) independent of protein pre-coating. Significantly more cells adhered to polystyrene tissue culture plates (PS). Only about half of these numbers were present on uncoated hydrophobic OTS surfaces, but cell adhesion on OTS was supported by serum proteins and fibronectin. The number of cells on PEG was as high as on OTS without any significant influence of protein pre-coating. 3T3 cells adhered well on OTS- and PEG-modified surfaces and to some lower

extent on oxidized wafers. In contrast, almost no MG63 and 3T3 cells adhered to uncoated hydrophobic fluorocarbon surfaces (HFS). However, cell adhesion to HFS was enhanced by serum proteins and strongly supported by fibronectin (Fig.1).

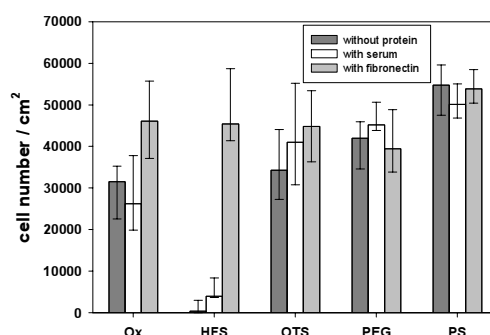


Figure 1: Adhesion of 3T3 mouse fibroblasts to SAMs pre-coated with fibronectin and fetal bovine serum.

A tight contact of MG63 cells to the HFS surface in the presence of fibronectin was visible on SEM images (Fig. 2). Cell attachment is specifically enhanced by FN on HFS, and FN probably adsorbed to HFS in a native conformation. Although HFS without adhesive FN is an incompatible surface, low amounts of FN detected on HFS supported the initial cell attachment.

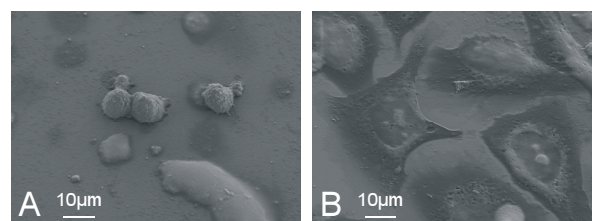


Figure 2: SEM images of MG63 osteoblasts on uncoated (A) and fibronectin-coated (B) HFS surfaces.

Conclusions

Substrate-dependent differences in protein adsorption displayed noticeable effects on cell adhesion. Pre-coating of biomaterials with adhesion proteins like fibronectin might be an effective tool to specifically improve cytocompatibility of fluorocarbon surfaces.

Acknowledgements

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STUDY OF THE DISSOLUTION PROCESS OF MAGNESIUM AND MAGNESIUM ALLOYS IN VITRO

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Introduction

Mg and its alloys are highly degradable and potentially useful for osteosynthesis application. Experiments made “in vivo” showed a significant increase in the osteo-mass around the implant [1]. However, the rate of corrosion is too high and these metals are not suitable for implantation. Results reported recently show higher corrosion rates of Mg alloys measured “in vitro” than “in vivo”. The modulation of the corrosion rate of Mg and Mg based alloys in the physiological environment has to be developed and investigated more deeply. Possibly ways should be the purification of Mg or variation in alloying composition or surface treatments [2]. Assessment of corrosion behaviour bases on electrochemical tests like voltammetric or impedance analysis[3]. In relation to the size of biomaterials as Stents f.e. and considering the numbers of variations of parameters of composition and surface treatments require a high performance electrochemical set up.

Aim

The aim of this work is to study the influence of the composition of organic fluids on the electrochemical response of Mg and Mg-alloys in an attempt to elucidate the causes of the differences between the dissolution processes that were carried out previously in NaCl containing solutions and the corresponding “in vivo” assays [4] using the MCS [5].

Materials and Methods

The dissolution processes that occur on pure Mg and Mg-alloy AZ31 surfaces were studied through polarization curves, open circuit potential measurements. Chlorid solutions of different concentrations (0,5%, 1% and 3,4%) and a standard buffer solution (PBS) were used as electrolytes. Albumin was also added to investigate the effect of proteins.

Results

The results showed that the corrosion process is strongly affected by the chloride concentration. The polarization curves exhibited a very unstable passivity zone and then the breakdown of passivity by localized attack. The passivity region was shorter at higher chloride concentrations. The corrosion potential records showed scattering values few seconds after the immersion of the Mg in the NaCl solution and PBS. The time after treatment and before measurement estimates the changes of the oxide layer, visible in the changes of the shape of the I-Ecurves, as shown in Fig.1.

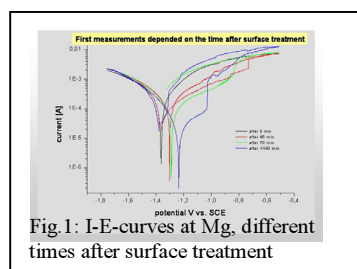


Fig.1: I-E-curves at Mg, different times after surface treatment

In the presence of proteins such as albumin, the passive zone is more clearly defined and the breakdown of passivity was more difficult.

The formation of a layer (that probably contained proteins) on the surface was detected through microscopic observations. The surface showed localized attack similar to that reported for aluminium in NaCl medium, where blisters that then exploded were found on the surface[6]. In the case of Mg, this process was enhanced when a pre-treatment at cathodic potentials was made and hydrogen was adsorbed within the surface layer.

Conclusion

It could be concluded that the differences reported for the corrosion “in vivo” and “in vitro” can be attributed on a great extent to the different chloride concentration between both conditions and to the absence of the organic components during “in vitro” experiments. Future work is focused on the detailed characterization and evaluation of the corrosion behaviour of Mg-alloys using the MCS. The letter could be demonstrate its performance for measurements at specimens based on magnesium and seems to be applicable for process controlling investigations.

Acknowledgements

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NOVEL PLGA 80/20 DS RELEASING MULTICOMPONENT IMPLANT

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Introduction

In our previous study [1] we have reported on developing DS releasing bioabsorbable rods. However, their drug release properties were unsatisfactory. We have thus assessed the use of sintering technique of enhancement of drug release in the current study.

Materials and Methods

Melt extruded PLGA 80/20 rods were compounded 8 wt-% DS. Three different components were produced by self reinforcing (SR) some of compounded 8 wt-% DS rods and sterilize some of the SR-rods. These three different rods were sintered with heat and pressure to form one multicomponent rod. Thermal properties were analyzed using differential scanning calorimetry (DSC) to determine glass transition temperature (T_g), melting temperature (T_m) and heat of fusion (ΔH). Drug release measurements were performed using UV-Vis spectrophotometer. There were three different specimen groups: A1 constructed from even parts of components, B1 and B2 from 47 volume-% of compounded and 32 volume-% of SR, and 21 %-volume of sterilised SR rods. B2 specimens were sterilized. Five parallel samples of three different specimen groups (A1, B1, and B2) were measured first at 6 hour intervals, then on daily basis and later about three times a week. Mechanical strength was measured during two weeks, after which the components disintegrated each other.

Results and Discussion

Release rate consisted of three different phases: 1) sharp jump start peak, 2) second smoother peak, and 3) the last smooth peak (Fig 1). The form of the profile depended of the fractions of different components.

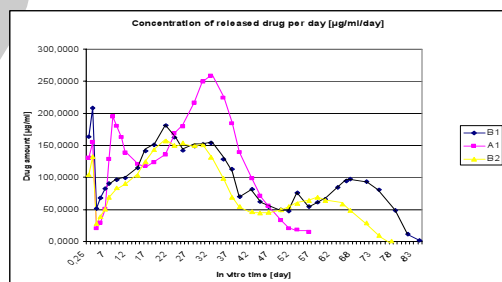


Fig. 1. Drug release profiles of the three specimen groups.

Released DS concentrations reached local therapeutic levels and maintained at that stage for 24-36 days

depending of the fraction of different components. All DS was released from the rods during 50- 70 days. Notable was also the accelerative effect of sterilization to the release.

The drug release profiles of initial components and sintered multicomponent differs from each other dramatically. It is easily seen that the drug release of multicomponent implant is more stable and begins earlier, which are the properties desired.

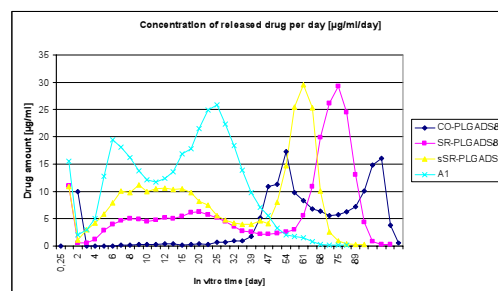


Fig 2. Comparison of drug release profiles of initial components to sintered multicomponent implant.

Initial shear strength was 82MPa and it decreased to 15MPa during two week in hydrolysis when after the components disintegrated. The mechanical attachment accomplished by sintering was sufficient although the components disintegrated too fast.

Conclusions

By sintering different PLGA/DS components, which have different release rates it is possible to construct a truly controlled release implant for bone fixation with anti-inflammatory properties.

Acknowledgements

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TISSUE MACROPHAGE RESPONSE TO CHRONIC ZIRCONIUM EXPOSURE

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Introduction

Titanium (Ti), and to a lesser degree, Zirconium (Zr), are employed as biomaterials in orthopedic and odontological implants. The direct interaction of these metals and the biological environment has been shown to take place in the interface zone. The metal oxide/fluid interface is relevant to the use of metallic implants in the biomedical field [1]. Although Ti and Zr differ quantitatively in their physicochemical properties (oxidation rate, interaction with water, crystalline structure and transport), both metals exhibit a passivating layer of oxide that prevents corrosion [2]. However, *in vivo*, no metal or alloy is completely inert. When the metal ions are released from the surface of implants they can migrate systemically, remain free in local intercellular spaces, or be taken up by macrophages. We have previously shown the presence of macrophages loaded with Ti particles (Fig.1) in the interface of human failed dental implants, as a marker of a corrosion process [3]. Moreover, we have found a significant increase in the generation of reactive oxygen species (ROS) in lungs of animals exposed to titanium dioxide (TiO₂) [4]. In a previous study, we also reported that 6 months post i.p. injection, the concentration of Zr dioxide (ZrO₂) in organs with macrophagic activity and the induction of ROS in lungs were lower than in the case of TiO₂. The aim of the present study was to evaluate the effect of ZrO₂ on tissues following chronic exposure (18 months).

Materials

Male Wistar rats were injected i.p. with saline solution (control (C), n=6) or with a suspension of 1.60 g ZrO₂/100g body weight (experimental (E), n=6) as described elsewhere [5]. Eighteen months post injection the following end-points were assessed: a) Zr in blood cells, b) deposits of Zr in liver, spleen and lung, and c) oxidative balance in lung homogenates. The latter was evaluated employing the total peroxyl radical trapping potential (TRAP) assay and by chemoluminescence initiated by tertbutylhydro peroxide (CL).

Results and Discussion

The presence of Zr was observed in blood phagocytic mononuclear cells and in the parenchyma of the organs analyzed (Fig. 2). The TRAP assay, which evaluates the content of low molecular weight hydrosoluble antioxidants, showed in the experimental group an increase in the antioxidant capacity as compared to control (E: 58 ± 8 vs C: 32 ± 8 μM Trolox/mg prot., p<0.05). CL, a marker of oxidative stress, revealed differences between both groups (E: 72100 ± 100 vs C: 50600 ± 120 cpm/ mg prot., p<0.05). Thus, animals under chronic treatment with ZrO₂ exhibited an adaptive

response that involved the mobilization of hydrosoluble antioxidants in response to the rise in the generation of reactive oxygen species. The persistence, at 18 months post-injection, of ZrO₂ particles in blood cells and in the organs of treated animals can be attributed to a slow clearance. It would be interesting to quantitate over time the amount of deposits in the organs. It is noteworthy that no apparent tissue damage associated to the particle deposits was observed.

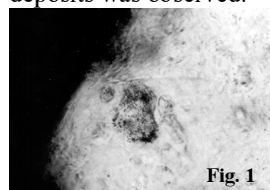


Fig. 1

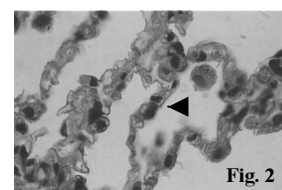


Fig. 2

Figure 1: Macrophage in the implant - tissue interface. Note the presence of particles in the cytoplasm. Orig. mag x1000.

Figure 2: Lung macrophage (►) loaded with ZrO₂ particles. Orig. mag. x1000.

Conclusions

The persistence of ZrO₂ particles in blood cells and in organs with macrophagic activity poses queries that warrant future studies on the assessment of long-term tissue response to zirconium. Because metallic implants usually stay in place for long periods of time, these data must be considered when those materials are employed in orthopedics and dentistry.

Acknowledgements

Grants: UBACyT 020 and CONICET PIP 6042.

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EFFECTS OF A NANOSTRUCTURED CALCIUM COATING ONTO MICROSTRUCTURED TITANIUM SURFACES ON OSTEOBLAST GENE EXPRESSION

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Introduction

Many studies have demonstrated the potential advantages of titanium (Ti) implants incorporating calcium ions (Ca) in enhancing osseointegration. The purpose of this study was to evaluate whether Ca-coating stimulates osteoblast differentiation on microstructured Ti implant surfaces by assaying osteoblast gene expression in vitro, which may affect the results of osseointegration in vivo.

Materials and Methods

Nanostructured CaTiO₃ coatings were produced on microstructured Ti surfaces by hydrothermal treatment using Ca-containing solution, this type of Ca-coating preserved the original micron-scaled surface properties, such as microstructure and microroughness, caused by blasting and/or etching in our previous study. Four different treated Ti surfaces were used in this study as follows: Group 1, #1200-grit abraded with SiC paper (Ma); Group 2, Ma with Ca-coating (Ma/Ca); Group 3, blasted with hydroxyapatite particles (RBM); Group 4, RBM with Ca-coating (RBM/Ca). The formation of the crystalline structure and chemical composition in the coatings were confirmed by thin-film XRD, XPS, and AES. MC3T3-E1 cells were cultured on different treated Ti surfaces. Real Time PCR was used for quantitative analysis of alkaline phosphatase (ALP) and osteocalcin gene expression at 7, 14, and 21 days.

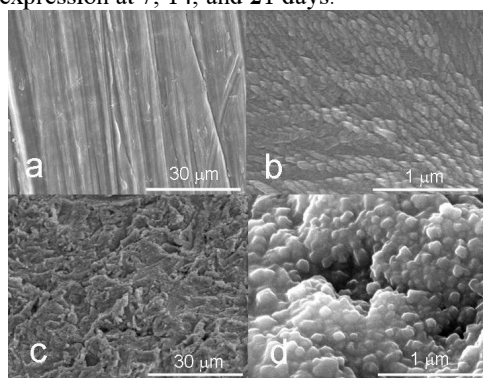


Figure 1. SEM images of Ma/Ca (a, b) and RBM/Ca surface (c, d) at magnification of $\times 1,000$ (a, c) and $\times 30,000$ (b, d).

Table 1. *Ra* values of Ti surfaces used in this study (Mean $\pm$ SD, μ m).

Ma	Ma/Ca	RBM	RBM/Ca
0.2131	0.2271	1.6741	1.7045
± 0.0218	± 0.0146	± 0.1309	± 0.1272

Results and Discussion

Real Time PCR showed significant increase in ALP gene expression in cells grown on RBM/Ca surfaces compared with other surfaces ($p < 0.05$), the peak activity of ALP appeared at 7 days (Fig. 2). Osteocalcin gene expression was significantly increased on microroughened surfaces compared with relatively smooth surfaces at 7 and 14 days ($p < 0.05$), RBM/Ca surfaces showed significantly increased osteocalcin gene expression compared to RBM surfaces at 14 days ($p < 0.05$; Fig. 2). Based on the results of this study, Ca incorporation onto the surfaces of microroughened Ti significantly increased ALP and osteocalcin gene expression in the early phase. In cells grown on relatively smooth surfaces, however, Ca-incorporation did not exhibit any stimulatory effect on osteoblast gene expression.

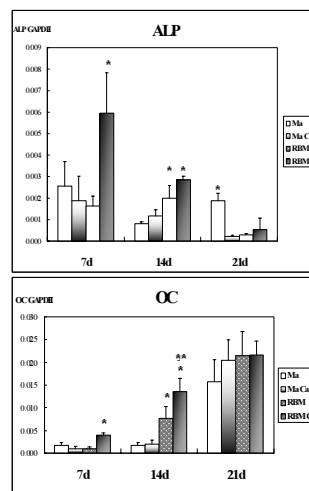


Figure 2. Expression of alkaline phosphatase (ALP) and osteocalcin (OC) gene expression on different Ti surfaces at 7, 14, and 21 days. *** $P < 0.05$.

Conclusions

The use of a nanostructured CaTiO₃ coating may have a synergic effect in enhancing osseointegration by stimulating osteoblast differentiation on the surfaces of microroughened Ti surfaces in the early phase.

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FIBRONECTIN ADSORPTION AND SUBSEQUENT CELLULAR ADHESION TO SELF-ASSEMBLED MONOLAYER PRESENTING IONISABLE CARBOXYLIC END-GROUPS

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Introduction

Cell adhesion to synthetic surfaces plays critical roles to numerous biomedical and biotechnological applications. The physicochemical properties of the underlying substrate, topography, wettability, chemistry, surface energy, modulate protein adsorption in term of adsorbed species density and conformational state. This study investigates the relationships between surface properties, protein adsorption and cellular "activity" of CHO cells on SAMs with different ionisable carboxylic end-groups treated with fibronectin.

Materials and Methods

Commercial glass cover slips and oxidized ATR silicon crystals were used as silica substrates. The silica substrates were cleaned using the standard RCA procedure. This produces a negatively charged and hydrophilic silica surface with a contact angle inferior to 10° (noted bare silica). The C₆H₅-terminated SAMs was obtained from grafting 1-phenyl 15(trichlorosilyl)heptadecane. Photo-oxidation by UV-ozone treatment of the C₆H₅-terminated SAMs permits to obtain the mixed C₆H₅-COOH-terminated SAMs. The COOH and COO⁻-terminated SAMs were obtained by the hydrolysis of COCl-terminated SAMs with an alkyl chain length of respectively 10 and 6 carbons. The physico-chemical surface properties of the different SAMs were characterized using water contact angle measurement (determination of the pK_a (surface carboxyl)) and FTIR-ATR spectroscopy (analysis of carboxyl-terminated SAMs) and classified according to their hydrophobicity (carbon number chain length; water contact angle respectively): [bare silica support] (0; <10°) < [COO⁻] (6; 67°) < [COOH] (10; 70°) < [C₆H₅-COOH] (≈13; 76°) < [C₆H₅] (15; 90°).

Fibronectin (Fn) was purified from human plasma. Protein adsorption is followed during 2 hours at 50 µg/mL. The analysis of the amide I' band of the FTIR-ATR spectra enables the determination of both the amount of adsorbed protein and its secondary structure evolutions. CHO cell behavior: adhesion, spreading and actin cytoskeleton organization were followed on the different surfaces, with or without coating of Fn.

Results and Discussion

On the most hydrophobic surfaces such as [C₆H₅], [C₆H₅-COOH] and [COOH], the adsorption plateau is reached at 8 minutes of contact with the fibronectin solution. The effect of negative charges induces a slower adsorption as seen for bare silica and [COO⁻] supports.

However the fibronectin adsorbed amount is higher for the [COO⁻] than for the highly hydrophilic silica surface. Both hydrophobic and electrostatic interactions play a role in the Fn adsorption. Concerning Fn conformation, compared to the state in solution, the adsorbed molecules of fibronectin are the most altered by the pure hydrophobic support [C₆H₅]. The adsorption on hydrophobic SAMs induces a major loss in the peptide carbonyls involved in unhydrated β-sheets and a minor loss in random domains in favor to the formation of newly hydrated β-sheets and hydrated random domains. The more the fibronectin molecules are adsorbed the more the cells are adherent. For the cell adhesion the parameter of interest is more the adsorbed amount of fibronectin than the conformation of the protein. But the cell spreading is favored by the denaturation of adsorbed fibronectin as seen for [C₆H₅], or by greater adsorbed amount as such [COO⁻]. The cell spreading on fibronectin adsorbed on bare silica is very weak in comparison with the other fibronectin-coated SAMs, as for [C₆H₅-COOH] and [COOH].

Although the adsorbed Fn amount on hydrophobic support is less, the cell distribution on hydrophobic support is similar to that of [COO⁻]. The conformational change of Fn adsorbed on [C₆H₅] compensates the effect of the adsorbed amount of Fn.

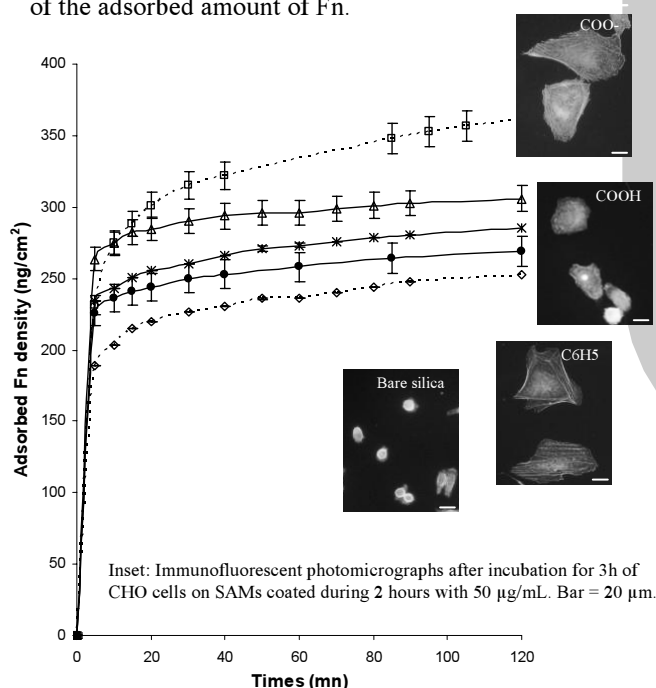


Figure: Fn adsorption onto SAMs as a function of time
◇: Bare silica, ●: C₆H₅, *: C₆H₅-COOH, △: COOH, □: COO⁻

MIMICKING THE NICHE – ADHESION OF HEMATOPOIETIC STEM CELLS ONTO EXTRACELLULAR MATRIX COATINGS

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Introduction

Bioartificial substrates may provide a powerful means to trigger stem cell fate decisions by imitating physical and biomolecular cues of tissue specific microenvironments. Towards this aim, we develop a set of surface-engineered model substrates to analyse the impact of spatial constraints and extracellular matrix (ECM) components on hematopoietic stem cells (HSC) grown in culture.

Materials and Methods

The stem cell niche is mimicked with respect to geometry by polydimethylsiloxane (PDMS) moulds containing cavities of 10 to 100µm diameter and 10µm depth. These carriers are coated with different ECM components and their supramolecular structures, respectively (tropocollagen, fibronectin, heparin, hyaluronic acid and co-fibrils of collagen I/heparin and collagen I/hyaluronic acid).

In initial adhesion studies on planar glass substrates HSC-ECM interaction was investigated by reflection interference contrast microscopy (RICM) to investigate the cellular contact zone in a quantitative manner. Covalent immobilization of ECM components was achieved by precoatings of poly(ethylene maleic anhydride) copolymers on the glass surfaces. CD133⁺ HSC from peripheral blood were grown on these carriers for 24h after fresh isolation or after 6d of cultivation to clarify influences of G-CSF mobilization of HSC. CellGro medium supplemented with low cytokine concentrations (SCF, FL3 and TPO at 10ng/ml each) was used to limit cell proliferation and differentiation.

Results & Discussion

Strong attachment of HSC to fibronectin with distinct adhesion areas was observed. In contrast to tropocollagen I and hyaluronic acid coatings, immobilized heparin and co-fibrils of collagen I/heparin and collagen I/hyaluronic acid also induced adhesion, however, with significantly smaller contact areas. In further experiments the attachment of HSC to fibronectin and heparin was analysed in detail. RICM and phase contrast microscopy were used in parallel to reveal quantitative numbers of the adhesion frequency and to unravel the involvement of $\alpha_5\beta_1$ -integrins in the adhesion of HSC to fibronectin and L-selectin as well as CD45 and SCF in the adhesion to heparin. It was found

adhesion to fibronectin, while blocking of L-selectin effectively prevented adhesion to heparin.

Conclusions

Ongoing experiments apply the above-listed ECM coatings to PDMS moulds with cavities of various dimensions to unravel combinatorial influences of spatial constraints and ECM on HSC homing, proliferation, and differentiation.

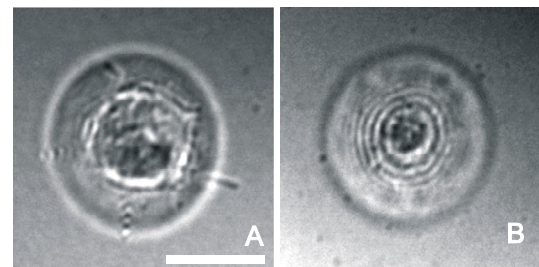


Figure 1: Adhesion characteristic of HSC after 24h of cultivation on matrix coatings (A fibronectin, B heparin) analysed by overlaid RICM and phase contrast images. RICM images show adhesion areas, phase contrast images visualize the overall cell shape. Scale bar: 5µm.

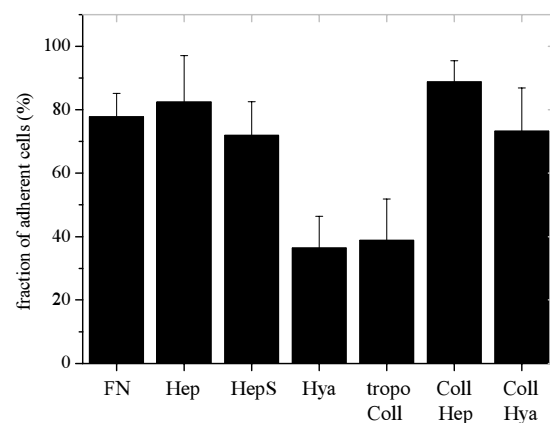


Figure 2: Fraction of adherent cells of HSC on several matrix components: fibronectin (FN), heparin (Hep), heparan sulfate (HepS), hyaluronic acid (Hya), tropocollagen I (tropoColl) and co-fibrils of collagen I and heparin (CollHep) or hyaluronic acid (CollHya).

COMBINING ADVANCED FORCE AND OPTICAL MICROSCOPY TECHNIQUES FOR BIOPHYSICS RESEARCH

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The range of biophysical applications for atomic force microscopy (AFM) continues to grow, as the advantages of high resolution imaging in physiological environments are combined with measuring and manipulating the structures under investigation. A key factor is the ability to combine AFM with advanced optical techniques, such as phase contrast, DIC and epifluorescent, TIRF or confocal imaging. This enables correlation of the high resolution structural information with specific labelling of active molecules. The BioCell allows full environmental control of samples on coverslips in liquid, for maximal optical resolution, single molecule imaging and spectroscopy, and cell studies.

The CellHesion development kit has been specially designed to extend the capabilities of AFM in cell binding and recognition assays, giving reproducible and quantitative analysis of cell-cell and cell-substrate binding forces. Single cells within a culture can be selected, attached to a flexible cantilever and subsequently allowed to adhere to a second, specific cell or region of substrate. Simultaneous information from advanced optical techniques gives insight into many additional cellular processes that occur on binding, such as changes to actin structure, calcium flushes, distribution of labelled proteins, or morphological changes.

A NEW PERSPECTIVE ON CELL-CELL ADHESION**K. Poole**¹, P.-H. Puech², D. Müller²¹JPK Instruments AG, Bouchestraße 12, 12435 Berlin, Germany²University TU Dresden, Biotec-Zentrum, Tatzberg 47-51, Dresden, Germanye-mail: poole@jpk.com

Cell-cell adhesion is a complex process that is involved in not only the tethering of cells, but also in cell-cell communication, tissue formation, cell migration and the development and metastasis of tumors. The complexity of cell adhesion and subsequent signalling, and the heterogeneity of the cell surface make it difficult to identify the contribution to cell adhesion by individual elements. Force spectroscopy, using an atomic force microscope, can allow one to distinguish

between interactions in the adhesion of a cell to a second cell or a coated substrate. In situ blocking experiments can help determine which surface molecules are involved in cell adhesion to a given surface. Such experiments will prove useful in distinguishing between effects of mutations on cell adhesion vs adhesion mediated signalling, as shown for the effect of the silverblick mutation in zebrafish gastrulation.

COATING OF TITANIUM IMPLANTS WITH EXTRACELLULAR MATRIX COMPONENTS

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Introduction

Coating of orthopaedic implants with extracellular matrix components appears attractive in order to enhance bone healing. Recent data from animal experiments suggest increased bone remodeling and bone formation around Titanium implants coated with type I collagen [1] and RGD peptides [2]. This study was designed to describe the cellular events of bone remodelling around Titanium rods with different organic coatings inserted into the rat tibia in a standardized experimental setting.

Materials and Methods

Titanium pins of 0.9mm diameter were coated with type I collagen (Ti/Coll), RGD peptide (Ti/RGD) or type I collagen and chondroitin sulfate (Ti/Coll/CS). Uncoated rods (Ti) served as control. The pins were inserted as intramedullary nails into the tibia of male adult Wistar rats. Six specimens of each group were retrieved at 4, 7, 14 and 28 days. Specimens were prepared for conventional histology, immune histochemistry and histomorphometry. For this purpose, specimens were embedded in MMA-based Technovit 9100N. All stainings and measurements were performed on cutting and grinding sections with the implant *in situ* [4]. Differences between the groups were analyzed with the Mann Whitney U-test, significance was assumed at $p < 0.05$.

Results

All types of implants healed uneventfully without visible adverse reactions. Mononuclear macrophages with staining against ED 1 appeared in higher average numbers around the Ti/RGD implants at day 4 and around the uncoated controls (Ti) at day 14 after implantation ($p < 0.05$). Osteoclasts and TRAP-positive mononuclear precursors were abundant around Ti/Coll/CS implants at day 7 ($p < 0.05$). A significant increase in osteopontin-positive osteoblasts were seen around Ti/Coll/CS implants at day 7 and around the Ti/Coll/CS and Ti/RGD implants at day 14 ($p < 0.05$).

At 4 weeks after implantation all pins were surrounded by a thin layer of newly formed lamellar bone. The amount of direct bone contact as percentage of the total implant surface after 28 days averaged 63.9% around Ti, 76.1% around Ti/Coll, 83.7%* around Ti/RGD and

89.5%* around Ti/Coll/CS implants (* $p < 0.05$ compared to uncoated Ti implants).

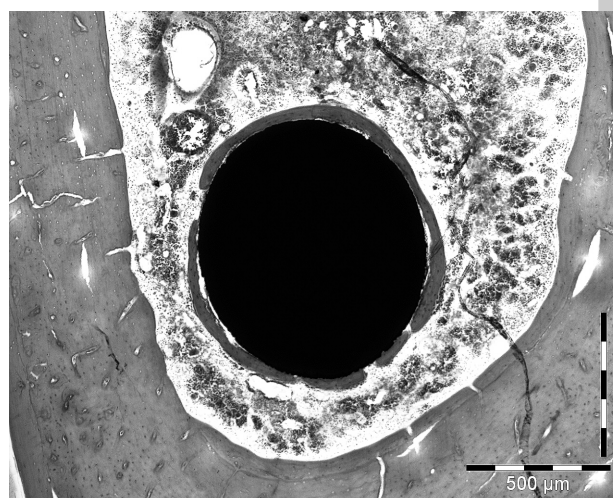


Figure 1: Micrograph of a Ti/Coll/CS pin 28 days after implantation into the rat tibia. The implant is surrounded almost completely by new lamellar bone (Goldner Stain, original magnification x50).

Conclusions

Compared to uncoated Ti, the addition of extracellular matrix components (type I collagen, chondroitin sulfate) or signalling sequences (RGD) significantly enhances bone remodelling in the early stages of bone healing around the implants. New bone formation at the implant surface after 4 weeks is increased significantly by the addition of either RGD or Coll/CS.

Acknowledgements

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CELL GROWTH ON LINEAR MICRO STRUCTURED SURFACES

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Introduction

Optimization of the electrode-nerve-interface is one of the key aspects in cochlear implant (CI) research today. A position as close as possible to the modiolus as well as low impedances are required for effective transmission of the stimulation signal from the electrode to the cochlear nerve. A few days after implantation there is an increase of impedance at the individual electrode contacts. This may be affected by the growth of connective tissue around the electrode, which is visible on explanted CI-electrodes. In addition, the electric stimulation signal becomes more unspecific. The starting point of our research project is therefore the purely physical modification of the interface between the implant and surrounding tissue, the electrode surface [1, 2]. The aim is to create an "unattractive" surface for connective tissue growth.

Material and Methods

The cochlear electrode contains platinum contacts in a silicone carrier. The silicon carrier (Cochlear Ltd, Sydney) comprises two types of silicone (LSR 30, HCRP 50). For our studies this silicone was provided with a polished and unpolished surface. In the first step gradual patterns were inserted into the material. The gradual structures presented in this study have a width of 1-10 µm and a depth of approx. 1 µm in linear configuration. To create these structures in the material with high precision and sharp edges we use the femtosecond laser technology. Cells were then cultured together with the materials processed in this manner. The use of GFP-marked cells (fibroblasts, neuronal precursor cells PC-12) makes it possible to observe cell growth even on non-transparent materials (e. g. electrode metals).

Cell growth can thus be observed on one sample over a period of several days, which makes this method particularly suitable for trials with only few samples.

Results and Discussion

The fibroblasts grow on the electrode materials. While the cell growth rate on platinum is similar to that of cells on glass, the cells on silicones show reduced growth. Laser structuring leads to further reduction of fibroblast growth.

The cell growth rate correlates with the width of the microstructure. The larger the width of structure the lower the cell growth.

This is true for silicone LSR-30 on an unpolished as well as on a polished surface. On silicone HCRP-50 with polished surface the structure seems to promote cell growth. On platinum the cell growth on structures with 4 to 7 µm width is reduced.

Neuronal precursor cells cultured and differentiated on micro structured materials show a strong guidance in neurite outgrowth on platinum but not so strong on both types of silicone (Fig. 1).

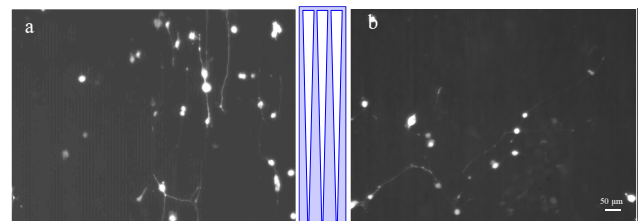


Figure 1: Neuronal Precursor cells on micro structured platinum (a) and on silicone LSR 30 (b), linear configuration of microstructure.

Outlook

GFP-marked fibroblasts are suitable as a model system for connective tissue cells. In further experiments structures of different size are to be tested on several electrode materials. The aim is to optimize the electrode interface, to reduce the connective tissue growth and to increase the electric contact to the neuronal target cells, the spiral ganglion cells.

Acknowledgements

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MONOLAYER DEGRADATION KINETICS OF POLYMER IMPLANT MATERIALS

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The study of enzymatic / hydrolytic degradation of polyesters is of great interest for many applications of these materials as implants, in controlled drug release and in tissue engineering. The monolayer degradation method can provide reliable degradation data of homopolymers, block copolymers and other molecules that form stable monolayers at the air-water interface. The reduction of the trough area covered with a monolayer of degrading material is measured thereby yielding the generation rate of water-soluble fragments. Only these fragments can participate in the erosion process of a bulk sample. Compared to bulk studies the monolayer investigations are fast and extremely small amounts of the materials are sufficient. As all cleavable bonds can be immediately accessed by water or enzymes, the method yields bond scission rates not influenced by diffusion phenomena observed in bulk samples. The potential of this method is demonstrated using data from poly- ϵ -caprolactone (PCL) homopolymers and statistical block copolymers of poly- p -dioxanone (PPDO) blocks and PCL-blocks with variable molecular weight and block ratio. A wide range of environmental conditions is probed, as e.g. enzyme concentration, pH and presence of water-soluble chemical and biological species. The PPDO-PCL copolymers are promising candidates for space memory implant applications, as sutures or stents [1]. The major advantage of these materials is that they get completely degraded inside the body and that they and their metabolites are known to be harmless with respect to foreign-body reactions.

Materials

The enzymatic degradation of polymer monolayers was studied using the lipase *pseudomonas cephalica* suspended in phosphate buffer. This enzyme selectively degrades the ester bond of the PCL blocks. The enzymatic degradation behaviour of the copolymers was compared with that in pure phosphate buffer solution. The study was performed on PCL homopolymers with a molecular weight of 10 kD, 43kD and 80kD and PPDO-PCL statistical block copolymers with PPDO blocks of 2kD and PCL blocks of 1,5kD and PPDO/PCL block ratios of $R = 33\% / 67\%$, $R = 50\% / 50\%$ and $R = 67\% / 33\%$.

Results and Discussion

The enzymatic degradation kinetics of the PCL homopolymers depends significantly on the molecular weight of the materials (see fig.1) and on the subphase pH. This kinetics meets the expectations of the arbitrary chain

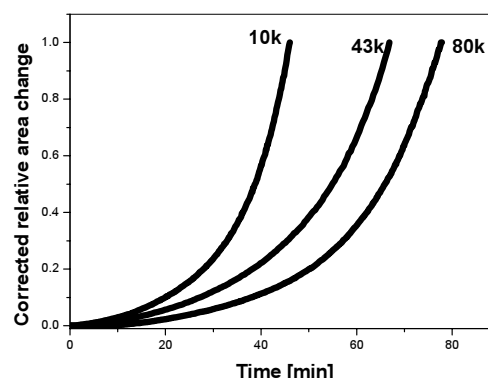


Fig. 1: Area reduction due to formation of water-soluble degradation fragments in PCL (parameter: M_w).

scission mode of the continuous distribution model [2]. The block copolymers of PPDO and PCL, however, show a completely different degradation behaviour yielding a linear area reduction curve. This kind of behaviour is usually observed in case of chain-end scission. In our case, the selective enzymatic cleavage of a short PCL-block results in the dissolution of this block together with the adjacent PPDO-blocks in the subphase. Again, a pronounced influence of the subphase pH on the degradation rate is observed. In bulk material this should lead to an “auto-inhibition effect”, as the degradation of polyesters results in acidic scission products.

Summary

The influence of environmental parameters on the degradation rate of implant materials can be systematically studied with monolayer experiments. A comparison of monolayer- and bulk degradation data (weight loss, X-ray diffraction data) gives insight into the diffusion kinetics of water and the penetration of enzymes into the polymers. For complex systems, such as phase segregated block copolymers, the separation of chain scission and diffusion phenomena becomes possible. Tuning of the degradation kinetics is possible by using block copolymers consisting of an appropriate percentage of slowly and fast degrading blocks.

Acknowledgements

We thank GKSS Research Centre for funding and Boehringer Ingelheim and Mnemoscience Aachen for sample materials.

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INTERACTIONS OF *STAPHYLOCOCCUS AUREUS* CELL WALL TEICHOIC ACIDS WITH MODIFIED TITANIUM IMPLANT SURFACES STUDIED BY QCM-D

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Introduction

Implant associated infections are often caused by biofilms. Wall teichoic acids (WTA) of *S. aureus* have been recently identified to play a mediating role in the bacteria-biomaterial interaction [1]. Prior to initial bacterial adhesion, implanted materials are conditioned by proteinaceous layers. This study investigates the adsorption and desorption characteristics of isolated WTA on different types of biomaterials as a function of macromolecular preconditioning (albumin, fibronectin) by means of an acoustic online sensor technique.

Materials

5-Mhz titanium coated quartz crystals were either surface modified by RFGD-plasma (water contact angle 35 deg), by DC-PEG (43 deg) or by amorphous teflon (122 deg). The crystals were integrated in a flow cell and used as acoustic sensors in a quartz crystal microbalance system (QCM-D), which allows detection of the dissipation signal (D) in addition to the resonance frequency (f) [2]. The online detected signals f and D indicate adsorbed mass and viscoelastic properties of the films, respectively. The flow cell was connected to a flow injection analysis system (FIA) that enables to inject analyte solutions into a permanent buffer flow (NaAc, pH 4.6). Human serum albumin (HAS, 1mg/ml) or human fibronectin (FN, 0.08 mg/ml) were injected in conditioning experiments prior to injection of wildtype WTA or dltA-mutant WTA (lacking D-Ala and more negatively charged than wildtype) (both 0.08 mg/ml).

Results and Discussion

The frequencies during adsorption-desorption of pure WTA were material dependent (Figure 1). Simultaneously observed small D shifts indicated low viscoelastic changes and allowed the calculation of adsorbed WTA masses after desorption: The masses were 134 ng/cm² on titanium, 100 ng/cm² on Teflon but -2 ng/cm² on DC-PEG, indicating major differences in the reversibility of that interaction between the tested surfaces. After preconditioning with HSA or FN a general decrease of the material dependency of the WTA interaction could be observed. Furthermore, the preadsorbed proteins induced a strong decrease of the adsorbed WTA mass and the WTA interaction was now

reversible on preconditioned surfaces. In contrast to the small D shifts observed during all experiments, the D signals monitored during WTA adsorption on FN indicated strong viscoelastic changes but total reversibility of D after WTA desorption. However, after the desorption phase, the sensors indicated a real mass loss that might be explained by desorption of FN in the presence of WTA. Differences between wildtyp WTA and dltA-mutant interactions were low. Thus, surface

charge may not play a predominant role for the WTA interactions on the tested surfaces.

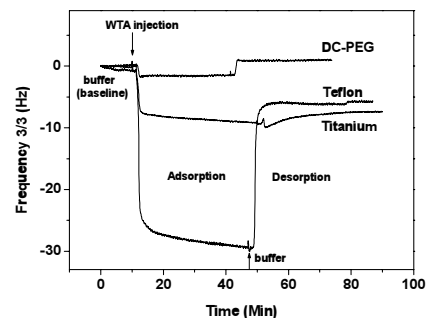


Figure 1: QCM-D frequency shifts indicating material dependent adsorption-desorption of teichoic acids.

Conclusions

WTA-surface interaction is dependent both on the type of biomaterial surface and the preconditioning by proteins. HSA and FN both seem to weaken the WTA-surface affinity. This offers possibilities to reduce *S. aureus* adhesion and biofilm formation on implants by e.g. biological modifications. The QCM-D/FIA setup was shown to be a useful online biosensor analytical system for the evaluation of new biomaterial surfaces.

Acknowledgement

Supported by fortune 1333-0-0 (University Hospital Tübingen, Germany).

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CLAMPED CIRCULAR ELASTIC DIAPHRAGMS AS SUBSTRATES IN CARDIOVASCULAR FUNCTIONAL TISSUE ENGINEERING

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Introduction

Cardiovascular functional tissue engineering relies among other factors on physiological mechanical stimulation of the construct for correct phenotypic differentiation of cultured cells. In order to simulate the cardiovascular environment in vitro, a device for applying physiological cyclic tensile strains to cell cultures has been developed and built. As a first step, the strain field generated by this device has been characterized using surface marker imaging techniques as well as in cell culture experiments. Furthermore, the influence of transitional strain regimes on microvascular endothelial cells was investigated. It was demonstrated that cells do not require precisely equibiaxial or uniaxial strain fields but rather respond to threshold values with respect to their orientational response.

Methods

The strain field on the cell culture membrane was visualized using fiducial markers and photographic analysis.

Then microvascular endothelial cells (CDC.HMEC-1) were cultured to either subconfluent or confluent monolayers on hydrophilized and collagen coated silicone substrates and strained for up to 144 hrs. at 11% average tensile strain. Endothelial cell response to strain and fluid shear stress were separated by selective disruption of the actin-/microtubule cytoskeleton. Cell orientation in the monolayer was visualized after 4% PFA fixation and subsequent silver nitrate staining. Cell orientation was evaluated with respect to the applied strain regime as a function of their radial position on the membrane.

Results

The generated strain field was shown to be homogeneous over the membrane area and conformed to the theoretical model for clamped circular membranes, indicating an equibiaxial strain situation at the centre of the membrane and a transition to uniaxial strain at the rim area.

Analysis of the orientational response of the microvascular cells confirmed two separate areas of predominant uniaxial and biaxial strain on the silicone membrane. However, cells were shown to tolerate certain deviations from these ideal strain situations up to certain threshold values.

Fluid shear stresses, generated by the design of the cell stretcher, were found to be highly turbulent in areas of

fluid deflection but elicited no significant orientational response from the cells.

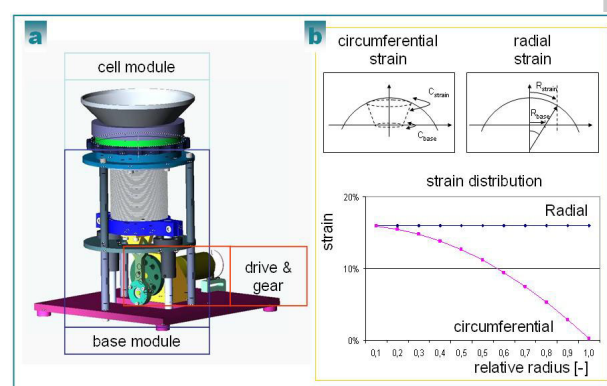


Figure 1: Fundamental design (a) and schematic strain field (b) generated by the cell straining device

Conclusion and Outlook

The cell straining device developed and built at the Helmholtz Institute was found suitable for application in functional cardiovascular tissue engineering.

In a second stage, the cell stretching device will be fitted with a module for applying an additional pulsatile pressure profile synchronized with the applied strain field that is currently under construction at the University of Hanover. Furthermore, investigations will be expanded into straining of 3D-constructs as well as the investigation of cells cultured on micro- and nanostructured surfaces.

Acknowledgements

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Zentrum für Biomedizinische Technik, Universität Hannover

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NITROGEN CONTAINING MONOMERS CAN INCREASE RESISTANCE OF HEMA TO PROTEIN ATTRACTION!

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Introduction

In the past two decades a great number of biomaterials have been developed for the progressive use as biomedical devices. However, to date, the problem of their biocompatibility has restricted their special use. It is essential for an implanted device to be able to avoid physiological rejection at the biological interface to which it applied. Rejection can be manifested in many ways depending on the biological environment of the implanted material. For example, a material rejected at a blood interface may cause thrombosis in a patient, whilst an inadequate contact lens material may promote tear protein and lipid deposition that will impair the quality of the lens and also give discomfort to the wearer. Despite of some limitations, poly(HEMA) has been widely used as a biomaterial for various application. It is shown that even a highly cross-linked poly(HEMA) matrix has relatively poor mechanical properties. Its uses are further restricted by relatively poor biocompatibility.

Methods

Three different nitrogen containing monomers, acrylomorpholine (AMO), N-Vinylpyrrolidone, (NVP) and N, N dimethyl acrylamide (NNDMA) were copolymerised with HEMA using bulk polymerisation at 60° C. The produced polymers were hydrated in distilled water with frequent changes for a period of two weeks and 1% ethylene glycol dimethacrylate (EGDM) was then used as cross-linker. The quantity of protein deposited on the materials after incubation in known concentration of protein solutions, was measured by U.V. at 280 nm.

Results and discussions

It was evident from the results obtained that NVP produces a copolymer that highly resist deposition of lysozyme and lactoferrin. It is known from the chemical structure that NVP carries a high positive charge, while NNDMA has a slightly positive charge due to the mesomeric effect of the methyl groups on the nitrogen. On the other hand, AMO is a hydrophilic monomer with low positive charge. The results (Table 1) are the mean values of three experiment carried out under the same conditions ($\pm$ SD). A typical representative adsorption diagram is shown in Figure 1. In this case, although NNDMA has introduced some positive charge on the polymer matrix, but it has adsorbed lysozyme (the positively charged protein, MW=14KD). This may be due to the small size and compact structure of the

enzyme. Among other proteins, ferredoxine (negatively charged with molecular weight similar to lysozyme) showed the highest adsorption on this positively charged copolymer.

Table 1: Monomer composition and protein adsorption.

Monomer	Protein deposited (mg/disk) ($\pm$ 0.002)				
(%)	Lysozyme	Albumin	Lactoferrin	Ferredoxin	Lactalbumin
NVP 10	0.030	0.026	0.025	0.034	0.030
NVP 30	0.022	0.036	0.018	0.044	0.041
NVP 50	0.012	0.039	0.010	0.049	0.048
AMO 10	0.052	0.027	0.033	0.035	0.028
AMO 30	0.073	0.033	0.051	0.032	0.022
AMO 50	0.082	0.041	0.054	0.029	0.01
NNDMA 10	0.033	0.023	0.024	0.033	0.031
NNDMA 30	0.030	0.029	0.018	0.040	0.036
NNDMA 50	0.027	0.028	0.016	0.046	0.038

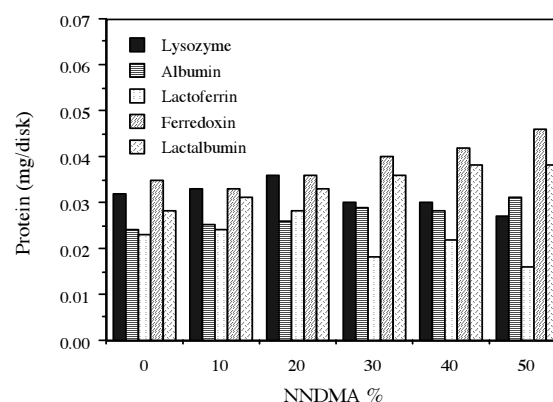


Figure 1: The absorption profile of proteins onto HEMA/NNDMA copolymers.

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INFLUENCE OF TITANIUM SURFACE MODIFICATIONS ON INITIAL PROTEIN/SURFACE AND CELL/SURFACE INTERACTIONS

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Introduction

Adhesion of macromolecules from blood and other body liquids leads to rapid formation of a conditioning film on the surface of implants during insertion. Physico-chemical surface properties like wettability, surface free energy or charge influence adsorption, composition and conformation of proteins forming this conditioning film. The adhering proteins, in turn, will indirectly determine subsequent cell/surface interactions as well as inflammatory and immune responses of the host.

The effect of titanium surface modifications resulting in highly wettable, modified acid-etched (modA) and sandblasted/acid etched (modSLA) surfaces were investigated with respect to initial protein/surface and cell/surface interactions and compared to the respective standard surfaces (A and SLA, resp.).

Materials and Methods

Titanium grade 2 disks 1mm thick and 5 or 15mm in diameter were acid etched (A) or sand blasted/acid etched (SLA). Part of the samples was modified by storing (under protective gas condition) in isotonic salt solutions at different pH's (modA and modSLA) immediately after etching. Initial hydrophilicity was quantified by dynamic water contact angle analysis (DCA).

β -Thromboglobulin (β -TG) and iC3b-complex were determined by ELISA following incubation of the samples in fresh human donor blood for 1h at 37°C. Results of at least two independent experimental series were tested for statistical significance using Students t-test.

Results and Discussion

Hydrophilicity: The modified process substantially increased hydrophilicity of the surfaces. First advancing mean (standard deviation) water contact angles were e. g. 137° (4°) for SLA, but 0° (0°) for modSLA [1].

Blood/surface and cell/surface interactions: On all modified surfaces, thrombocyte activation measured as release of beta-thromboglobulin was significantly decreased (Fig. 1). A less pronounced, but also significant effect was observed for complement activation, iC3b-Complex. Here the mean concentration was diminished e. g. to 84% ($p=0,03$) on modSLA surfaces compared to the standard SLA reference.

On the other hand, both fibronectin adsorption and cell adhesion of osteoblasts were enhanced on modified hydrophilic surfaces [2]. These combined effects of the hydrophilic surfaces on initial blood- and cell/surface interactions may contribute to an accelerated bone apposition *in vivo*.

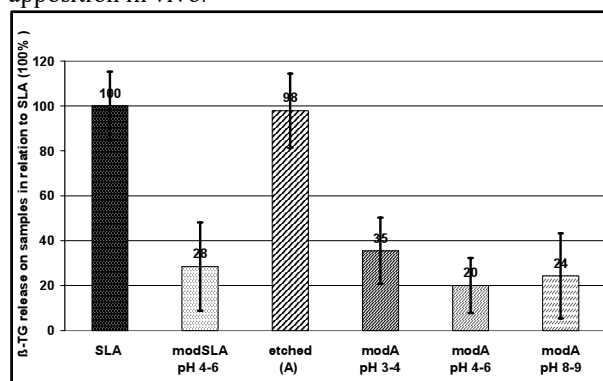


Figure 1: Mean amount and SD of β -TG-release on different surfaces

Conclusions

By chemical modification it is possible to overcome the initial hydrophobic behaviour of standard SLA surfaces. The process modifications influence initial blood/surface and cell/surface interactions *in vitro*.

The increased initial hydrophilicity and high surface free energy of these recent surfaces may also improve the healing process *in vivo*.

Acknowledgements

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EFFECTS OF COMBINED TREATMENT WITH ELECTRICAL STIMULATION AND DEXAMETHASONE ON SPIRAL GANGLION CELLS IN DEAFENED GUINEA PIGS

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Introduction

Cochlear hair cell loss leads to degeneration of spiral ganglion cells (SGC's). For Cochlea-Implant (CI)-funktion the SGC-density plays a decisive roll. Several studies indicate that the SGC degeneration can be reduced by electrical stimulation [1]. Dexamethasone (DEX) as an anti-inflammatory agent is able to reduce connective tissue growth along the implant. Therefore it could help to optimise the nerve-electrode interaction. The effect of a combination of both interventions simultaneously could lead to an improvement of CIs. To mimic the initial situation in a human patient we are interested in SGC survival in animals that have been deaf for a period during which a significant SGC loss occurs. We investigated the remaining cochlea function and degeneration pattern of SGC's in deafened guinea pigs treated with dexamethasone and continuous electrical stimulation.

Materials

Male pigmented guinea pigs (BFA) were systemically deafened by a co-administration of kanamycin (400mg/kg, sc) and ethacrynic acid (40mg/kg, iv) [2]. 21 days after deafening the left cochleae were implanted with an electrode/cannula device. The cannula was attached to a mini-osmotic pump (flow rate: 0,5µl/h) filled with either DEX (100ng/ml) or artificial perilymph (AP). The drugs were administered into the inner ear for 27 days.

The animals were divided into four groups. Two groups received DEX and two received AP. One group with DEX and one group with AP was additionally electrically stimulated via a portable stimulator (KHRI, Ann Arbor, Michigan, USA) for 24 days, 24h a day.

In all animals acoustically evoked auditory brainstem responses (AABRs) were recorded frequently. Electrically evoked ABRs (EABR) were recorded in the stimulated animals as well.

For all measurements and invasive interventions the animals were anaesthetised with xylazine and ketamine (10mg/40mg/kg, im).

48 days after deafening the cochleae were extracted, embedded in paraffin and 5µm sections were cut in a midmodiolar plane. After staining with haematoxylin and eosin 5 midmodiolar sections with a distance of 25µm were used for quantitative analysis of SGCs. The outline of each Rosenthal's canal profile was then traced and all SGCs in this area were counted to generate a SGC density, expressed as the number of SGC's in an area of 10.000µm².

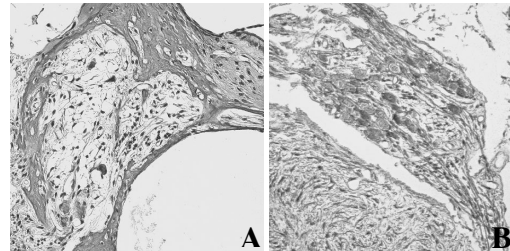


Figure 1: SGC's in deafened cochleae treated with DEX (A) and DEX+ES (B)

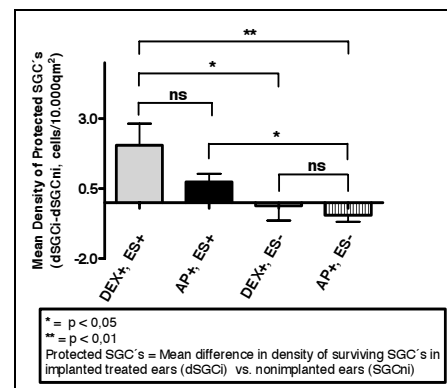


Figure 2: Effects of electrical stimulation (ES) and dexamethasone (DEX) on SGC's

Results and Discussion

Single DEX treatment has no effect on SGC-survival. ES treatment leads, as expected, to a significant protection of SGC's from 42% relating to the AP treated control group and from 30.5% compared to the DEX treated group. If ES and DEX are presented simultaneously the increase in SGC density is highly significant: this group showed a 77% higher mean SGC-density compared to the control group. Related to the only DEX treated group the DEX+ES-combination leads to a significant SGC increase of 65.5%. The SGC protecting effect of the combined DEX-ES-treatment is 34% higher than that one of single ES administration.

Conclusion

Dexamethasone enhances the protective effect of electrical stimulation on SGC's.

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Acknowledgements

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ASSOCIATION OF ANTIBODY RESPONSE AGAINST POLYMER MATRIX AND SEROLOGICAL CYTOKINE SECRETION FOLLOWING IMPLANTATION OF VASCULAR PROSTHESIS

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Introduction

The inflammatory response following implantation of biomaterials is assumed to be a major reason for bio(in) compatibility and for possible implant failure. The most important cells in this context are phagocytes and their products, although T and B lymphocytes are also detectable at increased levels. Recently we have investigated the humoral immune response after short and long-term implantation of polymeric biomaterials in animal models by detection of specific IgG antibodies. The present study aimed at differentiate the specific humoral immune response as well as the serological secretion of pro- and anti-inflammatory cytokines after repeated implantation/ explantation of vascular polyester prosthesis which differ in their impregnation/coating substances.

Materials

Experimental design: 40 male LEW.1A rats (age 100 days) were randomly divided into five groups (8 animals each) which received one segment (8mm x 8mm) of the following implants: G-I: collagen impregnated polyester graft (Dacron[®], Hemashield); G-II: Gelatine impregnated polyester graft (Vascutek[®]); G-III: albumin impregnated polyester graft (Bard[®]); G-IV: PTFE (Gore[®]); G-V: controls (sham operated). Blood samples for antibody detection and cytokine analysis were drawn from the retro-orbital sinus pre-operative and on experimental days 8, 15 and 22. On day 23 the segment was removed and cryopreserved for further immunohistochemical analysis. Two rats died after the third blood sampling (group I and II). **Antibody detection:** For polyester IgG antibody detection a polymer particle enzyme immunoassay was developed using a homogenized non-coated prosthesis (Microvel[®]) as target. Antibodies against PTFE (Gore prosthesis) were detected using PTFE prosthesis fixed in a MINIFOLD 96 PW (Schleicher & Schuell, Germany) as target. IgG antibodies against the prosthesis coating/impregnation were detected using bovine collagen type I, gelatine or human serum albumin (HSA) solid phase coated. **Cytokine analysis:** Rat interleukin-2 (IL-2), IL-4 and IL-10 serum concentration were measured by CytoSet[™] Antibody Pairs Kits (BioSource Germany GmbH). **Immunohistochemical analysis:** Serial cryostat sections of different explants and the surrounding tissue were prepared for examining macrophages by the monoclonal anti-ED2 antibody.

Results

In comparison to control G-V, the median anti-polyester IgG binding of G-II and G-III was significantly enhanced on day 15 after implantation ($p < 0.01$). The highest median immune response against the polymer matrix was obtained in G-II and G-III, where all animals were found to be antibody positive on day 22. In contrast, only 57.1% (4/7) rats of G-I had anti-polyester IgG on day 22. No rat of G-IV developed significant IgG antibodies against the PTFE matrix and all control rats of G-V were antibody-negative tested against both polyester and PTFE. Thus, the humoral immune response against the polymeric matrix can be differentiated as follows: Vascutek[®] > Bard[®] > Hemashield[®] > Gore[®]. As expected in G-III, a significant induction of anti-HSA antibodies was seen already on day 8, which did not correlate with the anti-polyester IgG of the individual rats. No anti-collagen and anti-gelatine IgG antibodies were detectable in rats of G-I and G-II, respectively.

The serum concentration of anti-inflammatory, B cell-associated IL-10 reveals a significant elevation on experimental day 22 in comparison to day 1 in rats of G-I, G-II, and G-III ($p = 0.012$) but not in G-IV ($p = 0.674$). Pro-inflammatory IL-2 was only significantly enhanced in G-III on day 22 vs. 1 ($p = 0.012$), the rats with a striking humoral immune response against both, the polyester matrix and the prosthesis impregnation. These findings were confirmed by immunohistochemical analysis demonstrating an intensive accumulation of ED2-positive cells in the peri-prosthetic tissue in rats with polyester prosthesis, especially in those of G-III, whereas comparably few cells were seen in rats implanted with the PTFE prosthesis.

Discussion and Conclusions

The study clearly demonstrates that the humoral immune response against the polymeric matrix of vascular polyester prosthesis is associated with a pronounced IL-10 response as well as a massive accumulation of inflammatory cells in the peri-prosthetic tissue of recipients. Moreover and most interestingly, a pro-inflammatory IL-2 response was found only in animals with a striking humoral immune response to both, the prosthesis matrix and the coating/impregnation substance. Therefore the determination of serological markers as antibodies and cytokines might serve as an additional parameter for evaluation of biocompatibility.

RGD-PEPTIDE-CONTAINING DEXTRAN COATINGS FOR TITANIUM IMPLANTS

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Titanium and its alloys are bioinert implant materials with excellent biomechanical properties widely used in bone and joint reconstruction. The integration of these materials into the surrounding tissue is strongly influenced by the implant surface structure and chemical composition. A promising approach to improve the tissue-implant interface resulting in an optimum implant integration consists in the immobilisation of cell adhesion-promoting molecules onto the implant surface. A well known motif of many extracellular matrix proteins playing an important role in cellular growth, differentiation and proliferation, is the Arg-Gly-Asp (RGD) peptide sequence. Different strategies are published for the immobilisation of the RGD sequence onto implant surfaces including physisorption, direct covalent fixation, or integration into RGD-releasing implant coatings.

Here we report on the design of a new biodegradable coating system for titanium implants to which cell-adhesive peptides like the RGD sequence can be covalently attached.

Materials and Methods

Glass samples sputtered with titanium (thickness: 100 nm) and polished TiAl6V4 specimens were employed as model substrates for implant materials. The metal surfaces were pre-treated by water steam plasma activation and subsequent treatment with isopropyl tri(N-ethylene diamino)ethyl titanate (ITET). In the next step carboxymethyl dextran (CMD, degree of substitution = 0.8) was coupled onto the activated surfaces using conventional carbodiimide (EDC/NHS) coupling methodology. A nonapeptide with a CYGGRGDTP amino acid sequence was used as cell-adhesive molecule. The peptide was immobilized by treating the substrates with 2-(2-pyridinyldithio)-1-aminoethane/EDC/NHS followed by peptide addition. X-ray photon spectroscopy (XPS) analysis was performed after each reaction step.

Mouse fibroblast 3T3 cells were used for cell adhesion experiments. An immunofluorescence staining of vinculin combined with 4',6-diamidino-2-phenylindole (DAPI) staining was applied to detect the cells and to study the formation of focal adhesion sites. Based on these techniques, the average cell spreading area of 3T3 cells seeded onto titanium substrates and the percentage of the area covered by cells at different coatings were determined.

Results and Discussion

Titanium substrates were coated with a functional CMD layer using an amino group containing titanate as adhesion promoter. The free carboxyl groups of CMD were partly transformed into 2-pyridinyldithio-ethylamide

moieties which react with the free SH-group of a cysteine-terminated nonapeptide containing the RGD motif. Coating of the titanium surface with CMD and peptide attachment as well could be confirmed by XPS analysis. The established reaction pathway has the advantage that peptides can be selectively immobilised via disulfide bridges without using protecting groups for the different functional groups of the peptide.

Using immunofluorescence staining the average spreading area of 3T3 cells seeded onto titanium surfaces coated with CMD (Fig. 1, Ti-CMD) and RGD-containing CMD (Ti-CMD-RGD) were determined in comparison with uncoated titanium substrates (Ti).

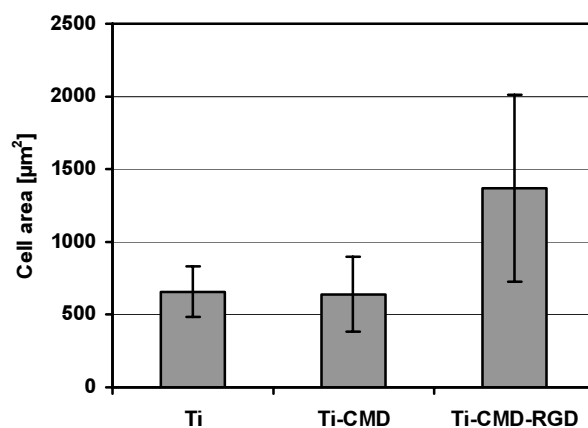


Figure 1: Influence of different coatings on the average spreading area of 3T3 cells 3 h after seeding on TiAl6V4 samples.

It could be shown that the average cell area of cells spread onto RGD-containing CMD coating is clearly increased compared to pure titanium and CMD coating as well. Similar results were found determining the percentage of cell covered substrate surface that includes the number of adherent cells.

Conclusions

A novel coating system for titanium implant materials based on a titanate adhesion promoter and carboxymethylated dextran was established. The functional CMD coating offers versatile possibilities for the immobilisation of bioactive molecules able to modulate the processes of cell adhesion and tissue formation at the implant surface.

Acknowledgements

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DETECTION OF *IN VITRO* CULTIVATED AUTOLOGOUS CHONDROCYTES *IN VIVO*

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Introduction

The aim of our study is to answer the question: What happens under *in vivo* conditions with autologous cells, which are used in reconstructive surgery for filling defects or for covering implants to suppress inflammatory reactions? Will these cells stay in place and become integrated in the surrounding tissue or are they simply opsonised? To follow the fate of the implanted autologous cells it is essential to distinguish these cells from the surrounding tissue in the living animal. Therefore, we employed non-invasive MRI-technology for the detection of specifically labeled cells after implantation in guinea pigs.

Material and Methods

Primary chondrocytes were isolated from cartilage of the guinea pig concha and expanded in culture dishes up to three passages as described earlier [1]. For *in vivo* studies cells were seeded onto the surface of a porous PE (MEDPOR).

For labeling of chondrocytes Iron-nanoparticles, Endorem (GUERBET), fluidMAG-D-/12, fluidMAG-D/Q, and targetMAG-N (Chemicell) of different size and polarity were tested. As a lipophilic tracer the fluorescence marker DIL_{C18} (Invitrogen) was used in different concentrations. The viability of the chondrocytes was estimated by trypan blue exclusion. For *in vivo* tracing, the cells were first labeled with Fe-particle (30µl/sample) before seeding. DIL labeling (10µg/sample) was done 2 days before implantation of the samples into the guinea pig. The pigs underwent MRI-examinations up to 2 month after implantation. At the end of the MRI-measurements the animals were sacrificed and used for histological examination. Fe-particles were stained by Prussian blue and analyzed by light microscopy. For fluorescence microscopical analysis the specimen were stained with DAPI and the signal of DIL_{C18} was detected with the G2A-Filter at 575nm.

Results and Discussion

To follow the fate of autologous cells after implantation into the donor by MRI-technique, Fe-nano particles of different size and polarity had been used to label chondrocytes *in vitro*. The best results were found for Endorem and fluidMAG-D/12 at a concentration of

30µl/ml of cell culture medium *in vitro*. Presence of Fe-particles will lead to a signal intensity reduction in a T₂-weighted MRI. Our aim was to detect Fe-labeled chondrocytes at the PE-surface *in vivo* by such a signal reduction. Our first results showed a loss of signal intensity of Fe-labeled cells seeded PE-particles. Although we could detect a signal intensity difference between Fe-labeled and unlabeled sample (Figure 1), further experiments have to be performed to increase the signal intensity differences between labeled and unlabeled samples to enable a quantitative analysis.

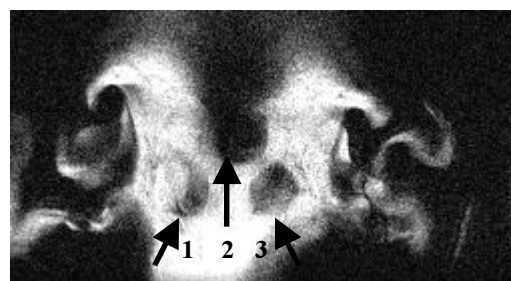


Figure 1: MRI of a guinea pig implants of PE, seeded with chondrocytes and labeled with Fe-particles 1 month after implantation (1-unlabeled, 2-fluidMAG, 3-Endorem)

First results of our histological examinations revealed the presence of Fe-labeled cells near the PE-implant. However, the amounts of cells should be higher to get a stronger labeling and by that a clearer effect on the signal intensity in the MRI. The same is true for the found fluorescence signal.

In summary, it is possible to distinguish autologous cells implanted in the donor from the surrounding tissue by labeling these cells with Fe-particles and/or fluorescence marker *in vitro*. For the *in vivo* visualization of Fe-labeled cells with the help of MRI further experiments have to be performed to optimize this procedure.

Acknowledgements

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USE OF GFP-EXPRESSED CELL LINE FOR TESTING BIOMATERIALS

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New method for testing biocompatibility and cytotoxicity of biomaterials is developed. The method includes use of GFP-expressed cell lines to visualize testing process and using PC for processing results. Combination of this method with traditionally used ones for investigation of cytotoxicity such as Crystal violet (CV) and MMT assay give more possibilities to test properties of biomaterials.

Materials**Cell line**

GFP-expressed PT-67 cell line was used as a model for detection of cytotoxicity properties of biomaterials used for bone transplantation. GFP gene was inserted into cell line PT67 (NIH/3T3-derived fibroblast cell line) by the method of retroviral transduction described by Yang et al., using system of RetroPack™ PT679 cell line (BD Biosciences) and plasmid pLEGFP-N1 (Clontech). For GFP-expressed cell selection transfected PT-GFP cells were cultured in the presence of 250 µg/ml G418 (Sigma) for 2 weeks.

Test materials

Cell tests were applied on two different materials: niobium-containing calcium phosphate glass ceramics 4Nk and acrylic bone cements ABC-HAp with 3-D polymer structure formed by copolymerization of mainly ethylmethacrylate and triethyleneglycol dimethacrylate.

Crystal violet cytotoxic assay

Sample was sterilized by UV-irradiation and then immersed in 2 ml of sterile simulated body fluid (SBF) for 24 h in 24-well plate at 37°C. SBF was substituted with 2 ml of the growth medium DMEM+ (supplemented with 2 mM glutamine and 10% fetal bovine serum) and the plate was placed into incubator in atmosphere of 5% CO₂ at 37°C. 24 h later sample was placed into a new well of 24-well plate containing 2 ml of PT-GFP cell suspension (104 cells /ml) in DMEM+ growth. The same concentration of PT67-GFP cell suspension in 24-well plate without sample was used as a control. Plate was incubated for 48 h in atmosphere of 5% CO₂ at 37°C. Further cells were fixed on the surface of sample by 1% glutaraldehyde and washed out sterile water. Attached cells were stained with 0.05% crystal violet solution for 15 min. The excess of dye was removed by washing sample twice with water. The amount of dye taken up by the sample was quantitated by extraction with the solution composed of 0.2 M citrate buffer (pH 4.2): ethanol 960 (1:1, v/v) and

subsequent OD540 measurement by Anthos HTII photometer. Obtained data and software PRISM (vers.3.00) were used to calculate the percentage of live cells in 1 cm² of sample surface against 1 cm² of the control well. Data of crystal violet staining presented in Tab.1 as percentage of live cells growing on the surface of the sample compared to control (100%).

The commercially available Aldrich-Sigma reagents were used in this study.

Light and fluorescent microscopy and data analysis

Stained sample as well as samples without cells and control well was pictured in UV light using NICON microscope Eclipse TE300 and camera NIKON Coolpix 990. Results presented in % of green fluorescence compared sample with control (100%) (Tab.1) Green fluorescence of sample surface was measured as cells green fluorescence minus sample background fluorescence

Table1. Comparison of CV assay and ImagePro data for calculation cytotoxicity of biomaterials

Sample/Method	CV (% of live cells)	Image (% of green fluorescence)
4Nk	362±2	118±7
ABC-HAp	82±15	83±3

We presume that the differences between CV and ImagePro data are related to the surface morphology of 4Nk, which is different from ABC-HAp. Cells grew in the open pores in surface of the sample, but this characteristic is not taken into account by Image Pro software. In the case of ABC-HAp sample PT-GFP cells grew on dense surface of the sample and were not able to penetrate into the sample and, thus, CV and Image data coincided very well.

We consider that use of GFP-expressed cell lines and processing of green fluorescent images provides not only data about cytotoxicity, but also additional information about permeability of the sample for cell growth without special experiments.

Acknowledgements

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EFFECT OF BIPHASIC ELECTRICAL STIMULATION ON EARLY BONE FORMATION AROUND IMPLANTS

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Introduction

Many studies and clinical applications have been recently tried for the early loading in the implant dentistry. In order for early loading to be stimulated, early bone formation around implant have to precede. This study was performed to investigate the effect of biphasic electrical stimulation as a method for early bone formation around implant

Materials

SLA implants were used in the experiments. SLA implants without electrical stimulation on one side of tibia of rabbit were considered as the control group, and SLA implants with biphasic electrical stimulation on the other side were as the test group. The electrical stimulation (biphasic current, 15 μ A 3000Hz) was applied for 1 week. Total 4 rabbits were tested, and all animal was sacrificed at 3 weeks after implant insertion. Histological specimens with H&E and Masson Trichrome staining were made and the bone contact rate with implant (% bone contact area/total implant surface on one side) was histomorphometrically evaluated using image analyzer.

Results and Discussion

A new bone formation was observed around implants in the experimental group after 2 weeks, whereas there was no new bone formation in the control group. The new bone formation began not only from the cortical bone around implant neck area but also isolated from the implant surface. The vessels were more observed in the experimental group than in the control group. The bone contact area (%) with implant after 4 weeks was more in the test group (43.9%) than in the control group (13.8%), which was statistically significant ($p=0.0006$).

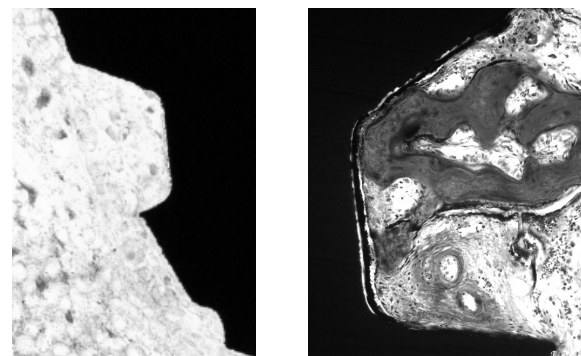
Conclusion

This study suggests that biphasic electrical stimulation is effective in the early bone formation around implant. It can be an alternative method in clinical application for the early loading of implant.

Table I: The bone contact area with implant (%)

Call for papers	Contact area (%)	SD
Biphasic electrical stimulation	43.93	18.04
control	13.77	11.84
$p = 0.0006$		

Figure 1: Bone formation around implant in the control group and in the test group..



Control

Biphasic electrical stimulation

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DISULFIRAM INHIBITS SMOOTH MUSCLE CELL PROLIFERATION

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Introduction

In-stent restenosis represents one of the most frequent long-term complications after implantation of coronary stents [1]. Prevention of in-stent restenosis by drugs released from Drug-Eluting Stents (DES) that inhibit SMC migration and proliferation is an attractive therapeutic approach. The drugs should be of low systemic toxicity and can be embedded in a polymer coating on the stent surface. The present work focuses on the antiproliferative drug disulfiram (TETD) [2]. TETD shows antiproliferative activity on various cell types, induces apoptosis, inhibits the transcriptional activator NF- κ B and inhibits a number of enzymes by complexing with metal cations or by reaction with functional sulfhydryl groups. Based on the available data it seemed worthwhile to investigate the potential of TETD for prophylaxis of in-stent restenosis.

Materials and Methods

The biological influence of TETD on the cell viability (MTS assay) and proliferation (BrdU assay) of those cell types relevant for in-stent restenosis was tested. Specifically, the responses of aortic smooth muscle cells (A7r5) and aortic endothelial cells (BFA) to TETD were determined. L929 mouse fibroblasts were chosen as controls. In addition, the influence of TETD on PDGF induced smooth muscle cell migration was assayed in a Boyden chamber. The influence of TETD on cell morphology was followed by transmission electron microscopy, and by immunohistochemical staining of microtubuli. Hsp70 was detected by an anti-Hsp70 antibody on western blots and served as a marker for TETD induced stress responses.

Results and Discussion

At concentrations equal to or above 10^{-7} M TETD inhibits smooth muscle cell viability and proliferation (Fig. 1) almost completely, whereas smooth muscle cell migration is gradually impaired by increasing TETD concentrations. In contrast, endothelial cell viability is not affected by TETD and proliferation is impaired at 10^{-6} M TETD concentration or higher. Smooth muscle cell proliferation is 10 times more sensitive to TETD than endothelial cell proliferation. This effect is also

reflected by tubulin depolymerization that occurs at lower TETD concentrations in smooth muscle cells than in endothelial cells. TETD does not induce a general stress response as no major change in Hsp70 expression can be seen. The status of tubulin polymerization is strongly affected by high concentrations of TETD leading to complete depolymerization and loss of cellular structure.

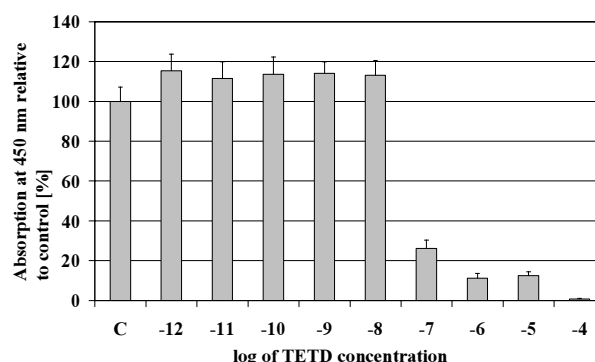


Fig. 1: Measurement of cell proliferation after exposure to TETD. Mean values are calculated from 6 parallel measurements.

Conclusions

TETD affects smooth muscle cell proliferation, viability and migration but does not impair endothelial cell viability. Therefore it is suggested to utilize TETD as a protective drug in Drug-Eluting Stents to prevent in-stent restenosis.

Acknowledgements

The work was financially supported by the German Federal Ministry of Education and Research within the program NBL3 (FKZ 01 ZZ 0108).

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NEURONAL DIFFERENTIATION INDUCED BY TRANSGENIC CELLS EXPRESSING NEUROTROPHIC FACTORS ON THE SURFACE OF COCHLEA IMPLANT MATERIALS

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Introduction

Although the advances in hearing aids and cochlear implants have been dramatic, these technological solutions can only be considered a compromise until cellular regeneration or replacement of the effective tissue can be achieved. To get closer to this goal we first want to improve current cochlear-implants by an optimized connection of the electrode-carrier to the surrounding nerve tissue. Primary aims are the protection from degeneration of the existing nerve cells (spiral ganglion cells) and additionally the stimulation of neuronal progenitor cells [1]. It has been shown that the external application of neurotrophic factors (NTF), e.g. GDNF, BDNF, NT-3 increases the survival rate of spiral ganglion cells in-vitro and in-vivo [2, 3].

Materials and Experiments

The protective effect of several neurotrophic factors on the survival of spiral ganglion cells and their potential to induce differentiation in neuronal progenitor cells was investigated. Murine Fibroblasts (NIH-3T3) were transfected via a lentiviral system to generate cell lines expressing several neurotrophic factors (GDNF, BDNF) and GFP under the control of a tetracyclin regulated promotor. The genes for the neurotrophic factors were arranged monocistronic or via an IRES-element bicistronic with GFP. As the bicistronic expression of GFP was insufficient we focussed on the monocistronic vectors for further experiments. The expressed and secreted neurotrophic factors were measured in the cell supernatant via an ELISA assay. Additionally we determined their biological activity by analyzing their potential to induce differentiation in PC-12 cells (rat pheochromocytoma cells). The supernatant of the GDNF producing cells was added to a growing culture of PC-12 cells and the induction of neurites was observed over a period of 10 days.

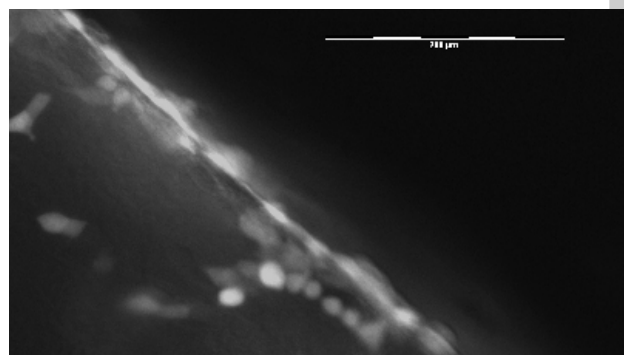
Results

Neurite outgrowth in PC-12 cells was observed beginning from day 2. Most of the cells showed clear outgrowth till day 10. As shown in table 1 the percentage of outgrowing cells and also the length of the neurites was strongly enhanced when the cells were cultivated with the supernatant of the GDNF producing cells, indicating the biological activity of the produced GDNF. Furthermore, it was possible to cultivate the transfected fibroblasts on silicone used for cochlear implants.

Table 1: PC-12 cells cultivated with/without supernatant of GDNF producing cells.

Cultivation time (days)	GDNF	Cells with neurites (%)	Average length of neurite (µm)
3	--	3.85	9.98
3	++	22.3	23.71
10	--	9.9	13.7
10	++	28.23	95.3

Figure 1: Transgenic fibroblasts cultivated on silicone (LSR30).



Conclusions and Outlook

Transgenic fibroblasts produce biological active GDNF. PC12-cells are suitable as a model system for neural differentiation and are useful for the investigation of differentiation of neural progenitor cells on implant surfaces.

In the following experimental phases transgenic fibroblasts will be co-cultivated on the surface of cochlear implants with PC12-Cells and also spiral ganglion cells.

Acknowledgements

This work was supported by the German Research Foundation (DFG), SFB 599.

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ANALYSIS OF OXIDATIVE STRESS IN ENDOTHELIAL CELLS GROWN ON Ti6Al4V

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Introduction

Endothelial cells (EC) are involved in wound healing after implantation by formation of new blood vessels and expression of pro-inflammatory molecules. Thus, maintenance of viability and functionality of EC is important in implant acceptance and long-term implant stability. EC in contact or proximity to implant surfaces are subjected to reactive oxygen species (ROS) produced by inflammatory cells and formed during cathodic corrosion of e.g. titanium alloys. While the oxidative stress response of EC to ROS is well studied, the effects of corrosion-induced oxidative stress in EC are unknown. Therefore, we compared the reaction to H₂O₂-induced oxidative stress of EC grown on Ti6Al4V and on cell culture plastic (polystyrene/PS).

Materials

Human dermal microvascular EC (HDMEC) were grown on PS and Ti6Al4V and exposed to 0.25/0.5 mM H₂O₂. Proliferation rate was analyzed by counting nuclei and detection of the proliferation marker Ki67. Cytotoxicity was measured with lactate dehydrogenase (LDH) and MTS conversion assays. Oxidative stress induction was studied by dichlorodihydrofluorescein (DCF, a ROS-sensitive dye) and determination of reduced glutathione levels.

Results and Discussion

Comparison of EC growth rates after H₂O₂-treatment (24 h) on Ti6Al4V and PS revealed a decrease in the overall cell number and reduction of proliferating cells (shown by Ki67 staining) to almost the same extent on both materials. In contrast, EC grown on Ti6Al4V displayed lower metabolic activity (MTS conversion assay) 24 h after exposure to H₂O₂ indicating a higher cytotoxic effect. Higher cytotoxicity by H₂O₂ on Ti6Al4V was shown by the LDH assay (higher LDH-release by EC on Ti6Al4V).

This higher toxicity could be attributed to an increased formation of ROS (shown by an increased fluorescence of DCF) in EC grown on Ti6Al4V compared to EC grown on PS (data not shown). Induction of oxidative stress in the EC cultured on Ti6Al4V was confirmed by GSH-measurement (depletion of the GSH pool is a sign for oxidative stress); while H₂O₂ induced a slight in-

crease in the GSH level in EC on PS the GSH amount was reduced in H₂O₂-treated EC on Ti6Al4V (Fig. 1). Interestingly, GSH concentrations in EC grown on Ti6Al4V were significantly lower even without H₂O₂-treatment. This might indicate the presence of permanent oxidative stress in EC on Ti6Al4V due to formation of ROS during the corrosion processes. This effect might be elevated on Ti6Al4V surfaces due to the production of radicals in the reaction of H₂O₂ with the TiO₂ layer [1].

This finding is in agreement with the increased lipid peroxidation and decreased antioxidant enzyme activity in the tissues surrounding titanium implants in laboratory animals [2].

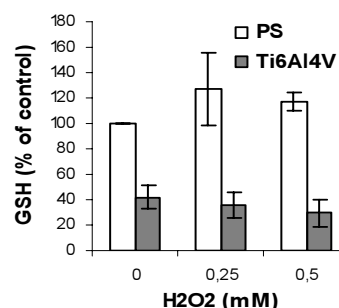


Figure 1: GSH levels in non-treated and H₂O₂-treated EC grown on Ti6Al4V compared to cells grown on PS.

Conclusions

In this study we demonstrated that EC grown on Ti6Al4V displayed higher cytotoxic effects to H₂O₂-treatment compared to cells grown on PS. Furthermore, steady-state- and H₂O₂-induced oxidative stress was more pronounced in EC on Ti6Al4V surfaces.

Acknowledgements

This work was supported by the German Research Foundation (DFG, KI 601/4-1).

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MODIFICATION OF ENDOTHELIAL CELL GROWTH ON TRICALCIUM PHOSPHATE BIOMATERIALS BY COCULTURE WITH OSTEOBLASTS

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Introduction

Porous beta-tricalcium phosphate (TCP) composites are finding use as a biomaterial for bone due to their high osteoblast cell-compatibility and biodegradability. The primary reason for this is that the physico-chemical composition of ceramics made of calcium phosphate is very similar to the mineral constituents of human bone. However, both in vivo analysis of TCP implants and in vitro studies with osteoblasts growing on TCP show that bone formation and osteoblast cell growth are strongly influenced by the purity and porosity of TCP.

The functioning of a tissue requires a healthy vasculature. Thus, neovascularization or angiogenesis is an essential step in the colonisation of porous biomaterials and osteointegration.

Endothelial cells are the primary cells making up the vasculature. Little is known of the influence of beta-TCP and porosity on the attachment, growth and gene regulation of endothelial cells on these materials and how osteoblasts effect these functions. The goal of this study was to examine endothelial functions on beta-TCP and in coculture with osteoblasts.

Materials and Methods

Cells: Primary human endothelial cells from the skin (HDMEC) and primary osteoblasts were isolated and cultured as previously described [1, 2]. The human osteoblast cell line MG-63 was obtained from culture collections and cultured in primary osteoblast medium.

Biomaterials: Two pure-phase beta-TCP ($\text{Ca}_3(\text{PO}_4)_2$) biomaterials in disc form (10mm x 2mm) were examined (generous gift from Curasan AG, Kleinostheim, Germany). TCP1 had a pore size of 0.1 - 50 μm (porosity 35%) and TCP2 had a pore size of 0.1 - 500 μm (porosity 65%).

Analysis of endothelial growth and functions:

Endothelial cells were added to biomaterials as described, coated with fibronectin or mixed with osteoblasts and then added to materials. Endothelial cell attachment, growth and spread on the material was visualised by confocal microscopy after staining with calcein-AM, labelling cells with qdots, or after fixation and staining for PECAM-1. Angiogenesis conditions were as previously described [2]

Results and Discussion

Osteoblasts grew very well on both TCP-1 and -2. Endothelial cells attached poorly to TCP-1, exhibited unusual cell morphologies with little cell-cell contact,

showed no signs of replication and individual cells detached with time although some cells survived up to 28 days. Coating with fibronectin or other extracellular matrix proteins did not improve the attachment or result in the replication of cells on TCP-1. Unexpectedly, endothelial cells attached, spread (proliferation) and exhibited a normal morphology on the larger-pored TCP-2 and survived for up to at least 28 days. Thus, the porosity of this biomaterial plays an important role in endothelial cell survival, and the inability of cells to survive on the smaller-pored biomaterial may be due to the inability to form cell-cell contacts.

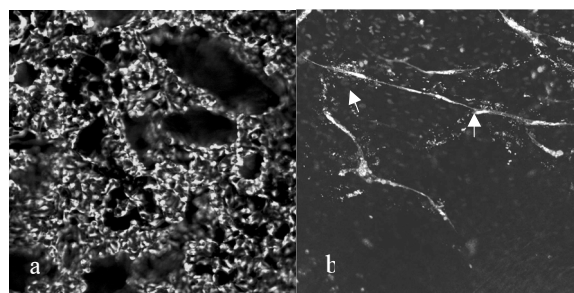


Figure 1. a is a confocal image of HDMEC on TCP-2, b is IF of a coculture of HDMEC and osteoblasts showing microcapillary-like structure formation (arrows).

Under conditions stimulating angiogenesis, no movement of cells to form microcapillary-like structures was observed. Surprisingly, in cocultures of endothelial cells with osteoblasts, with increasing time and without the external addition of angiogenesis-stimulating reagents, pathways of endothelial cells formed with features similar to microcapillary-like vessels. Thus, coculturing cells was sufficient to induce microcapillary-like structures by endothelial cells.

Conclusions

Both porosity and coculture with osteoblasts influences endothelial adhesion, growth, survival and angiogenic potential on biomaterials with identical chemical composition.

Acknowledgements

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THE INFLUENCE OF DIFFERENT SURFACE MACHINING TREATMENTS OF RESORBABLE IMPLANTS OF DIFFERENT MAGNESIUM ALLOYS – A PRIMARY STUDY IN RABBITS

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Introduction

Resorbable implants do not require further surgery to be excised. The aim of several current research projects is the development of resorbable implants which can also be used in weight bearing bones. Their resorption has to be adapted to fracture healing. Former studies indicate that magnesium alloys are qualified for usage as resorbable implants [1]. Gas production is known to occur during the corrosion of magnesium alloys [2]. Surface properties influence biocompatibility and therefore implant success [3]. The aim of this primary study was to examine the influence of different surface textures on the resorption of magnesium-calcium-implants in vivo.

Materials

3 x 5mm cylinders of different magnesium-calcium-alloys (calcium concentrations of 0,4%, 0,8%, 1,2% and 2,0%) were differently machined which led to plane (surface roughness 3,65µm), rough (surface roughness 7,85µm) and sandblasted (surface roughness 32,7µm) surfaces. Three implants of each concentration-surface-combination were produced (totally n = 36) and implanted into the spongy-cortical passage of the medial femur condylus of New Zealand White rabbits. For six weeks the animals were examined daily and x-rayed weekly. After euthanasia the bone was explanted and scanned in a microcomputed tomography.

Results and Discussion

The implants were well tolerated by the rabbits, neither lameness nor signs of pain occurred. Wound healing was nearly without complications. At the place of insertion all implants induced obvious callus formation. They showed different stages of resorption. Plane implants revealed structural changes only in the border area (Fig.1). Sand blasted cylinders showed the highest degree of resorption which could be seen by microcomputed tomography (Fig.2) and the clinical occurrence of gas production. This gas production was only visible in the sand blasted implants. Their degradation was very fast and the organism was not able to remove the gas. But the small gas bubbles disappeared within the course of four weeks. Rough implants showed the most irregular degradation. Some featured high

degree resorption, others had signs of resorption only at border areas.

Conclusion

The rougher the implant surface the faster the implants' resorption. Long time experiments which have already started must show which surface will be adequate for resorbable implants.

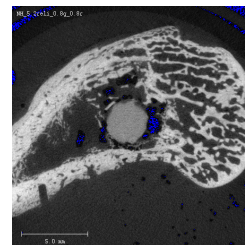


Figure 1: plane implant, resorption with structural changes in the border area



Figure 2: sand blasted implant, completely structural loss

Acknowledgements

This study is part of the collaborative research centre 599 (SFB599), which is sponsored by the DFG (Deutsche Forschungsgemeinschaft).

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LOCAL TISSUE REACTIONS AND THE DEVELOPMENT OF PROSTHESIS-SPECIFIC ANTIBODIES AFTER IMPLANTATION OF VASCULAR PROSTHESIS IN PIGS

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Introduction

It is well-known that the implantation of a biomaterial such as vascular prosthesis results in a local inflammatory response at the site of the implantation. In addition to that, processes like formation of a neointima at the contact between the body tissue and the implant as well as vascularization are part of the body's reaction toward an implant. Furthermore, we found specific antibodies against the prosthesis material and the coating substances in earlier studies in rats and mice. The aim of the present study was to evaluate the local reaction as well as the humoral response following functional implantation of Dacron®-based vascular prosthesis in a pig model.

Materials

Quantitative evaluation of the local tissue reactions. Juvenile female pigs with a body weight between 24 and 29 kg (n=12) were randomized into three groups. A part of the infrarenal aorta of each animal was replaced with segments of three different bovine collagen impregnated Dacron®-prosthesis (length 4cm, diameter 8mm; primary porosity: C – <50 cm³/min/cm²; M – 160-360; T – 500-750). The prosthesis and a sample of the surrounding tissue were removed from one animal per group on day 14, 21, 28 and 116 after OP, respectively. The tissue samples were evaluated by microscopy to assess the thickness of the neointima. Furthermore, the expression of beta-1-integrin, an important marker for cell-cell and cell-biomaterial interactions, in the area around the implant was examined by immunohistochemical staining. To get quantitative results from microscopy, digital image analysis was performed using the image analysis software KS 400, Release 2.0 (Kontron Elektronik GmbH, Eching, Germany).

Evaluation of the humoral immune response. A second group of pigs (n=24) was randomized into three experimental groups (M, C, T) and underwent the same OP procedure as described above. Two animals died after surgery, resulting in group sizes of n=7 for the M and T group and n=8 for the C group. On the day of the operation as well as 10, 17, 24 and 116 days after implantation, blood samples were collected. They were analyzed for antibodies using enzyme-linked immunoassays with (a) native bovine collagen type I, or (b) particles prepared by homogenizing a polyester prosthesis, as targets.

Results

Table I: Thickness of the neointima (µm)

Prosthesis	Experimental Day			
	14	21	28	116
M	236	809	988	1513
C	208	702	825	1113
T	126	764	966	1058

Table II: Expression of beta-1-integrin (%)

Prosthesis	Experimental Day			
	14	21	28	116
M	38,5	31,0	13,2	10,5
C	32,3	17,1	17,2	12,2
T	25,6	24,2	12,6	8,3

Table III: Prevalence of antibodies (%)

Prosthesis	Experimental Day			
	10	17	24	116
Target: prosthesis material				
M	57,1	57,1	28,6	0,0
C	37,5	37,5	37,5	25,0
T	28,6	28,6	28,6	14,3
Target: native bovine collagen Typ I				
M	28,6	57,1	57,1	85,7
C	37,5	25,0	12,5	62,5
T	71,4	100,0	57,1	57,1

Discussion and Conclusions

Differences regarding the tissue reactions at the site of the implant could be demonstrated for the three prosthesis types which were examined in this study. The most pronounced neointima formation as well as the highest level of beta-1-integrin expression in the early phase of the study was observed for the M-prosthesis. Interestingly, this prosthesis type also had the highest prevalence of prosthesis-specific antibodies during the early phase of the study as well as a steady increase of the prevalence for collagen-specific antibodies over the whole period, indicating a possible correlation between the humoral and cellular reactions. However, additional studies, for example the morphological evaluation of other inflammatory cells, are required to reveal the nature of both components of the immune response against implants in more detail.

NANOPOROUS SILICA COATINGS AND THEIR MODIFICATION

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Introduction

The development of novel bone replacement materials for the middle ear, where implants are used for the replacement of damaged ossicular chains, is guided by special requirements. Apart from optimized sound transmission properties, an attenuated bioactivity is required. Classical bone replacement materials are optimized for a fast and strong incorporation to fulfil the mechanical functions of bones. In case of the middle ear prosthesis, however, strong ongrowth of bone would lead to mechanical fixation of the implant, which is detrimental to sound transmission. One of the aims of this project therefore is the attenuation of the bioactivity of highly bioactive materials.

Materials

In order to prepare samples to be used in cell culture tests, glass slides were in a first step coated with nanoporous silica, using a dip-coating procedure. In a second step, these high surface area coatings were modified by linking functional organic molecules to the surface, in order to investigate the influence of chemically different surfaces on cell adhesion, growth and proliferation. Finally, such nanoporous coatings were also produced on standard ceramic biomaterials. Interactions with cultured cells were examined in DMEM with 10 % FCS under standard cell culture conditions (37 °C, 5 % CO₂) using the osteogenic murine embryonic mesenchymal progenitor cell line, C3H10T1/2. Cell adhesion, spreading and proliferation on coated glass slides were examined semi-quantitatively by microscopic examination. For examination of coated bioglass samples the cells were labeled with eGFP and the cells were examined with a UV fluorescence microscope.

Results and Discussion

Silica coatings with nanostructures in the range from 5 to 12 nm were prepared. Although the size of the nanostructures showed no significant influence on cell behaviour, the high surface area of these materials can be used to effectively modify the chemical properties of their surfaces. This was obvious in the cell culture studies (Figure 1). Cell adhesion was good on glass and nanostructured silica surfaces, as was cell spreading and proliferation. In contrast, chemical modification of the

surface with trimethylsilyl groups (or other chemical groups, not shown here) lead to decreased cell adhesion and proliferation. This shows that the nanostructured surface can be modified effectively to improve the surface properties of implant materials in applications where an overgrowth with cells is not desirable.

Conclusion

Nanoporous silica coatings with small nanostructures can be produced on glass and on standard biomaterials. The functionalization of the nanoporous silica coatings provides an effective means to control cell adhesion, growth and proliferation.

Acknowledgements

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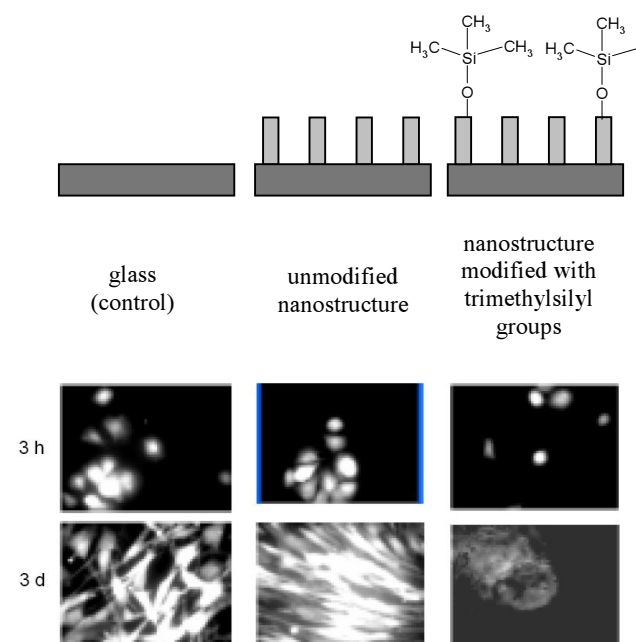


Figure 1. Cell cultures after 3 hours and 3 days of incubation on standard glass slides, on a silica nanostructure and on a trimethylsilyl-modified nanoporous silica surface.

DEVELOPMENT OF BIOACTIVE GLASS FIBER REINFORCED STARCH-POLYCAPROLACTONE COMPOSITE

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Introduction

For bone regeneration and repair in many cases combinations of different materials are needed. In many applications it is useful to combine biodegradable polymers with osteoconductive materials, as for instances, bioactive glass. One other aim is to try to improve mechanical properties of polymer matrix by means of its reinforcing. Thus, the aim of this work was to develop a bioactive glass fiber reinforced starch-polycaprolactone composite.

Materials and Methods

The composite was produced by extruding thick sheets from starch-polycaprolactone (SPCL - 30/70 wt%) blend. Sheets were cut and heat-pressed in layers with bioactive glass fiber mats to form composite structures, with different combinations: 6xSPCL+5xBaG, 3xSPCL+6BaG, 3xSPCL+4BaG, 3xSPCL+2xBaG. 3xSPCL and 6XSPCL were used as nonreinforced controls. Thermal, mechanical, and degradation properties of the composite were studied. In addition, the real amount of glass in the composites was determined using simple burning tests.

Results and Discussion

A strong endothermic peak indicating melting at about 56°C was observed from DSC analysis. TGA showed that thermal degradation of SPCL

started in 300°C with degradation of starch and continued in 380°C with degradation of PCL. Mechanical properties of reinforced composites were considerably better than the properties of non-reinforced composites. Reinforcing increased shear strength by 50%, tensile strength by 52%, and bending strength by 67%. However, mechanical properties of the composites dropped during two weeks in hydrolysis to the same level of non-reinforced controls. The degradation time of SPCL, as expected, was long; during the 6 weeks hydrolysis the mass decreased only about 5%. Degradation will of course occur faster at a later stage. The amount of glass in the composites remained the same for the 6 weeks period of hydrolysis.

Conclusions

It is possible to enhance initial mechanical properties of starch-polycaprolactone by reinforcing it with bioactive glass fibers. However, mechanical properties need to be further improved for allowing long-lasting bone applications.

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DEPOSITION OF HA COATINGS BY MICROPLASMA SPRAYING

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Introduction

Hydroxylapatite (HA) is one of the most important materials for human hard tissue implants because of its close resemblance to the chemical composition of teeth and bones. Atmospheric plasma spraying (APS) is widely used to deposit HA onto implants due to its high deposition rate and economical advantages [1]. However, due to high temperatures of plasma jet and rapid cooling of sprayed particles, the degradation of HA is inevitable during spraying, which involves the formation of tetracalcium phosphate, tricalcium phosphate, amorphous calcium phosphate and calcium oxide. In order to achieve a long life, the HA coating must have limited dissolution after implantation. This means that the degradation of HA during spraying must be minimized. Microplasma spraying (MPS) was developed only a few years ago. This spray process has a much lower plasma power and much smaller spray spots due to its narrow laminar plasma jet compared to a conventional APS process [2]. These process properties promise to minimise overheating of HA particles during spraying and allow using of finer HA powders. In this study, HA coatings were deposited by the new coating process.

Materials and Methods

A HA spray powder with a size distribution of -53 µm was deposited by a microplasma spraying system onto TiAl6V4 substrate at Surface Engineering Institute, RWTH Aachen University. The spray parameters were varied to investigate their influence on the coating build up and properties. The coatings were investigated in terms of their microstructure and crystallinity by optical microscopy, REM and X-ray diffraction analysis. Their bio-compatibility was evaluated by in-vitro tests which were carried out by Inasmet according to ISO 10995-5. Furthermore, cell proliferation and viability of human bone cells were assessed by flow cytometry, while activity of the alkaline phosphatase (ALP) enzyme was determined by fluorescence measurement.

Results and Discussion

The spray trials showed that the spray parameters electric current intensity, plasma argon flow rate, spray distance and carrier gas flow rate influenced significantly the deposition behaviour of the spray powder and the coating properties. Despite the lower plasma powers during microplasma spraying, dense coatings with a porosity of below 2 % could be produced. The coating crystallinity could be adjusted in

a wide range of about 50-90 %. In general, higher electric current intensities, lower argon flow rate and bigger spray distance led to lower coating crystallinities, because the powder particles were better melted. Compared to the established APS process, microplasma spraying can realize much higher crystallinities because the powder was much less overheated during spraying. Due to the small spray spots of 3-5 mm, this process is very interesting for more economically coating small parts such as dental implants.

The in-vitro tests showed a good compatibility of the coatings tested (See Fig. 1) concerning cytotoxicity. Cell proliferation and ALP expression resulted highly dependant on the coating properties, i.e. deposition parameters.

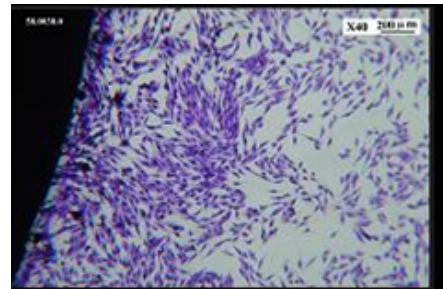


Figure 1: Cytotoxicity test sample on a HA coating produced by MPS.

Conclusions

The results show that dense HA coatings can be deposited by microplasma spraying. The coating crystallinity can be varied in a wide range. Compared to the established APS process, microplasma spraying can realize even higher crystallinities. The in-vitro tests showed that MPS coating performance can be at least as good as that of APS coatings. The new coating process can be very interesting for coating small parts such as dental implants with thin HA coatings.

References

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