Electrospinning of commercially available medical silicone rubber

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Introduction
Electrospinning is a well investigated technique for processing ultrafine thermoplastic fibres. The published techniques are mostly based on a polymeric solution and some on melt polymers. To improve heat and chemical resistance as well as gas permeability electrospinning of silicone rubber was aimed in this project. Possible applications may be hydrophobic membranes or coatings for a wide range of applications in clinical and experimental medicine.

Methods
To be able to adapt the process on a wide spectrum of silicones, commercial room temperature vulcanizing (RTV) silicone was used for process development. The chosen two component silicone (Silpuran® 2430, Wacker AG) was modified in viscosity and conductivity. Solvents, which have been used successfully for adapting viscosity and which led to a stable electrospinning process, were acetone and petrolether. Conductibility was tried to improve with NaCl and LiBr. Furthermore, the electrospinning process needed to be adapted to the curing properties of the silicone. A heated collector and infrared-lamps were evaluated for optimal curing parameters.

Results
Membranes have been electrospun successfully out of standard RTV-silicone. The jets respectively fibres have been spun in this project with diameters around 20 µm. Membranes with a thickness of 100 µm showed little improvement concerning hydrophobic properties and permeability. In addition, electrospun membranes showed a surface enlargement in comparison with pressed test specimens. The purpose of improving conductibility of the silicone-solvent by adding NaCl and LiBr was not reached so far.

Conclusion
The investigated technique showed the possibilities of an innovative processing method for silicones. With this project basics for electrospinning of silicones have been established for diverse applications, like membranes, coatings or other tissues. The process combined with silicone properties appears to be from special interest for the medical technology. Based on the presented results detailed research on the medical use of the processed silicones can be investigated in future.
Combination of drug delivery and stable biofunctionalization on bio-degradable implant surfaces via layer by layer deposition

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Abstract

Polyelectrolyte multilayer (PEM) films, established using the layer-by-layer (LbL) technique, are attractive for controlled drug delivery in order to improve tissue regeneration. For instance, the LbL-deposition provides the possibility to incorporate biomolecules via electrostatic interactions for drug delivery, which might be moreover combined with a stable biofunctionalization via covalent binding of biomolecules such as cell adhesion motifs to the outer layer. Here, the influence of covalent biofunctionalization of PEM deposited on poly(L-lactide) (PLLA) on the release profiles of avidin fluorescein isothiocyanate (avidin-FITC) incorporated as model drug within the PEM is demonstrated.

1 Introduction

Tissue regeneration combines several processes that have a different temporal progress. Consequently, tissue engineering strategies afford scaffolds specifically designed to mediate tissue-scaffold interactions by surface modification with bioactive ligands, or the integration of a drug delivery system. Besides several other synthetic biodegradable polymers, poly(L-lactide) (PLLA) has already been successfully used as scaffold material and as matrix for local drug delivery (LDD) systems because of its relatively good mechanical and manufacturing properties, as well as moderate biocompatibility [1]. However, the biomedical applications of PLLA, especially cell adhesion, are hampered to a certain extent by its low wettability. A promising approach to simultaneously increase the hydrophilicity and provide the PLLA surface with an LDD function is the surface modification via polyelectrolyte-multilayers (PEM), deposited by layer-by-layer deposition (LbL). Additionally, the outer layer of the PEM enables the covalent bonding of biomolecules, which can improve the tissue regeneration.

The principle of multilayer assembly is as follows: a solid substrate with a charged surface is immersed in a solution containing polyelectrolytes of opposite charge leading to deposition of a first polyelectrolyte layer via electrostatic attraction. The resulting coated substrate is immersed in a second solution containing polyelectrolytes of again opposite charge with regard to the substrate surface. By repeating these steps, alternating multilayer assembly is obtained. Due to the possibility to use a high variety of polyelectrolytes the properties of LbL films are tunable and thus embedded drugs can be released from the material in a predefined manner. Next to the embedding of drugs, the PEM provides the possibility to bind biomolecules covalently to the outer layer, such as cell adhesion molecules via crosslinking reactions.

In this study, we investigated the combination of embedding the model drug avidin-FITC within PEM deposited on PLLA with the covalent binding of the Arg-Gly-Asp (RGD) sequence on the outer PEM layer. In this case PLLA modified using the LbL deposition with chitosan as polycation, and polyacrylic acid (PAA) as polyanion, which turned out to be promising in previous studies, were examined [2]. The drug release behavior of the embedded avidin-FITC will be discussed in dependence of the covalent binding of biomolecules such as biotin-RGD using the crosslinker ethyl(dimethylaminopropyl) carbodiimide/ N-Hydroxysuccinimide (EDC/NHS) on the cover of the PEM system (Figure 1).

Figure 1: Schematic representation of multifunctional PEM using chitosan as polycation, PAA as polyanion deposited on PLLA, which combines the covalent binding of biotin–RGD and the incorporation of avidin-FITC for drug delivery.

2 Methods

All chemicals were purchased from Sigma Aldrich, Mallinkrodt Baker, SERVA Feinbiochemica, Thermo Scientific and Merck in p. a. quality or higher.

The planar PLLA samples (Resomer L210, Boehringer Ingelheim, Germany) were modified, using plasma etching (PE) electrode in an oxygen (O₂) radio frequency (RF) plasma generator (frequency 13.56 MHz, power 100 W, Diener electronic GmbH & Co. KG, Ebhausen, Germany) for 5 min. The polyelectrolyte multilayer films were fabricated as already described [3] via a robot (KSV Nima, Espoo, Finland) with the following dipping protocol: 5 min in chitosan solution (2 mg/mL in 25 mmol/L sodi-
um acetate buffer pH 5.0), 3 washes (10, 20, 30 s), 5 min in PAA solution (2 mg/mL in 100 mmol/L sodium acetate buffer pH 5.0), 3 washes (10, 20, 30 s), 10 min in avidin-FITC (40 µg/mL in 25 mmol/L sodium acetate buffer pH 5.0), 1 wash (10 s) and 5 min in PAA with 3 washes (10, 20, 30 s). This cycle was repeated to achieve 5 tetralayer films. Dipped foils were allowed to dry for 48 h at 4 °C.

The covalent coupling of biotin-RGD was carried out in two steps. First streptavidin was coupled by the crosslinking agent EDC/NHS. Therefore PEM-modified PLLA substrates were dipped into the crosslinker solution (0.04 mmol/L EDC/NHS in 100 mmol/L PBS-buffer pH 5.0) for 10 min. After the dipping process in the washing solution (PBS-buffer pH 5.0, containing 10 mg/ml BSA, 0.14 mmol/L Glycin and 0.01 w% Tween 20) for 30 min the system was dipped in the streptavidin solution (40 ng/mL in 100 mmol/L PBS-buffer pH 5.0) for 1 h followed by the washing step for 30 min. The amount of linked streptavidin was examined using streptavidin-HRP which can be analysed via ELISA. A 3,3',5,5'-tetramethylbenzidine (TMB) substrate solution, a soluble colorimetric substrate for horseradish peroxidase (HRP) enzyme, was added to the HRP conjugated PEM. In the presence of HRP, the TMB and hydrogen peroxide contained in the substrate solution react to produce a blue by-product. After addition of sulfuric acid (2 mol/L) the color changes to yellow, enabling accurate measurement of the intensity at 450 nm by means of the plate reader FLUOstar Optima (BMG Labtech, Offenburg, Germany) using a calibration curve. Biotin-RGD was then linked to the streptavidin-modified substrates by dipping in a biotin-RGD solution (40 µg/mL biotin-RGD in 100 mmol/L PBS-buffer pH 5.0) for 1 h. Afterwards the functionalized system was washed again. The amount of linked biotin-RGD was examined using biotinylated HRP, which can be analysed via ELISA as described above. Each step of layer by layer process was verified via Contact angles measurements by sessile drop method (Contact Angle System, OCA 20, Dataphysics Instruments GmbH, Filderstadt, Germany) and the morphological changes were examined in a XL30 (Philips, Eindhoven, The Netherlands) scanning electron microscope.

The release of avidin-FITC from the investigated PEM (Ø = 6 mm) in 350 µl basal medium (pH 7.4) at 37°C was measured by means of the plate reader FLUOstar Optima (BMG Labtech, Offenburg, Germany).

### Results

The surface morphology of PLLA is hardly changed after deposition of the alternated charged layer. Only with the addition of avidin-FITC as embedded biomolecule the surface got more structured (data not shown). The PEM surface containing avidin-FITC hardly changed after the addition of TLs (Fig.2). The PEM surface got again more structured with the covalent coupling of the biotinylated RGD (Fig. 2).
4 Discussion

The PEM surface morphology was characterized via SEM and it was detected that with addition of avidin-FITC the surface of the PEMs got rougher. Also the surface got rougher with the covalent coupling of streptavidin and biotin-RGD. These effects are probably due to the coupled biomolecules, which crystallize after the crosslinking during the drying process. Furthermore, the contact angle, detected during the layer by layer process for both biomolecules decreased, indicating a more hydrophilic surface and the deposition of layers on the un-activated PLLA. This decrease is most probably due to the more hydrophilic groups of the used polyelectrolytes. The covalent coupling of biotin-RGD influences the release of the embedded biomolecule avidin-FITC. The total amount of the released avidin-FITC was decreased by the coupling. This can be probably due to the fabrication process because during the dipping process for the covalent coupling on the cover of the PEM parts of the incorporated avidin-FITC can be already released. In further investigation the fabrication process will be optimized.

5 Conclusion

The release studies of avidin-FITC demonstrated that the covalent coupling of biotin-RGD seems to have an influence on the released drug amount, which can be most probably optimized by the dipping process. Furthermore, the dual functionality of the fabricated PLLA based PEM - PLLA (ChitIPAAI growth factor IPAA)2 streptavidinbiotin-RGD seems to be a promising coating for drug delivery systems which can combine for instance the attraction of endothelial cells (biotin-RGD) and their proliferation (release of growth factors) during the wound healing. Therefore, in future experiments the effect of this synthesized dual functionalized PEM using VEGF instead of avidin-FITC, as embedded drug, on human umbilical vein endothelial cell (HUVEC) behavior in vitro will be investigated.

6 Acknowledgement

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7 References


Figure 4: Avidin-FITC release from PLLA based PEM Bars show mean ± standard deviation of n = 3 experiments.
**In-vivo-model to evaluate the suitability of a biodegradable magnesium alloy (MgYREZr) for intraarticular orthopedic implants**

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**Problem statement**

The fixation of ruptured anterior cruciate ligaments is currently performed with interference screws made of titanium or biodegradable polymers. These materials are disadvantageous because of a second operation for implant removal or reported adverse effects. Magnesium alloys are promising candidates as an alternative implant material because of their biodegradable and biocompatible properties. Aim of this study was to investigate the effects of the resulting corrosion products of a magnesium alloy (MgYREZr) on the synovial membrane of the knee. Based on these results, the following study deals with the fabrication and testing of interference screws made of the magnesium alloy.

**Material and methods**

The animal experiment included a total number of 36 New Zealand White Rabbits that were divided into two implant groups with 18 animals each. In the first group a magnesium pin was used, and identically constructed titanium pins in the second group. The groups were subdivided into 3 groups with the implantation periods of 1, 4 and 12 weeks. The pins were implanted into the femoral notch of the left stifle joint of each rabbit. After the implantation periods samples of the synovial fluid and synovial membrane were examined histologically by a semiquantitative score.

**Results**

Clinically, all implants were well tolerated without any signs of inflammation or lameness. The radiological examination indicated no excessive gas formation and no alterations of the bone structure. The histological evaluation of the synovial membrane showed some mild inflammatory reactions after 1 and 4 weeks with a marked decline after 12 weeks. There were no statistically significant differences between the two implant groups.

**Conclusions**

The outcome of this study supports the theory that the tested magnesium alloy is suitable for intraarticular applications. Further animal studies are intended to investigate the influence on bone and tendon tissue.
Hydrogels with precisely nano-functionalized micro-topography for cell guidance

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Abstract

In vivo, cells encounter different physical and chemical signals in the extracellular matrix (ECM) which regulate their behavior. Examples of these signals are micro- and nanometer-sized features, the rigidity, and the chemical composition of the ECM. The study of cell responses to such cues is important to understand complex cell functions, some diseases, and is basis for the development of new biomaterials for applications in medical implants or regenerative medicine. Therefore, the development of new methods for surface modifications with controlled physical and chemical features is crucial. In this work, we report a new combination of micelle nanolithography (BCML) and soft micro-lithography, for the production of polyethylene glycol (PEG) hydrogels, with a micro-grooved surface and decoration with hexagonally precisely arranged gold nanoparticles (Au-NPs). The Au-NPs are used for binding adhesive ligands in a well-defined density. First tests were performed by culturing human fibroblasts on the gels. Adhesion and alignment of the cells along the parallel grooves of the surface were investigated. The substrates could provide a new platform for studying cell contact guidance by micro-structures, and may enable a more precise control of cell behavior by nanometrically controlled surface functionalization.

1 Introduction

Features of the extracellular environment co-regulate complex cell behavior and functions in vivo and in vitro. The signals can be of chemical nature, for example given by the chemical composition of the extracellular matrix (ECM), or physical, for example by the ECM stiffness and topography on nanometer and micrometer size scale. Cells are able to sense these signals, transduce them into intracellular biochemical signals, finally leading to biological cell responses [1]. Although many molecular details of these biological processes are not in detail understood, there is a long history on using micro-sized textures of surfaces to control adhesion and behavior of cells in contact with them [2-4]. Several techniques of surface modifications have been conceived. Very often, structures with sizes in the micrometer range have been used to study the behavior of different cell types such as fibroblasts or osteoblasts [2,5,6]. One well-examined example of cell response to surface topographies on micro-manufactured substrates is the so-called contact guidance. There, cells respond to topography by adapting their cell morphology or migration with respect to elongated surface features such as parallel grooves. This phenomenon was first reported by R. Harrison in 1914 [3]. Next to this phenomenon, there are other in vitro cell responses. Surface topographies can alter the gene expression profiles of cells and could be utilized as a signaling modality for directing differentiation. One impressive example is the reprogramming of somatic cells into pluripotent stem cells [7]. Cultivation of somatic cells on a surface with parallel microgrooves could partially substitute the effects of epigenetic modifiers and improved re-programming efficiency. While many previous studies have used micron-sized surface features produced by conventional technologies such as photolithography, improved methods for fabricating nanostructured surfaces have enabled novel studies in the past decade. Several reports have impressively demonstrated that even surface features with heights in the range of a few nanometers or small variation in adhesive ligand distance of a few nanometer can drastically influence the behavior of cells growing on such surfaces in vitro [8-11]. Since the size of the topographical structures influencing cells can be in the range of nanometer and micrometer, the combination of fabrication techniques promises the production of substrates that offer topographic cues with both appropriate length scales. One technique for producing polymer substrates with specific μm-sized surface topography is based on the well-established method of soft lithography. Invented in the mid-1990s it found many applications in biomedical research [12]. In a first step, a master wafer is produced by conventional optical lithography (Image 1). The desired lateral structure of the surface topography is transferred to a silicon wafer covered with photo resist by an optical illumination process and appropriate developing steps. The resulting surface topography is transferred to a polymer substrate, usually by casting a polymer solution of poly(dimethyl)siloxane (PDMS) on the master wafer and peeling it off the wafer after curing. The surface of the polymer substrate is now structured with the desired topography. Although such methods pro-
vide valuable surface modifications down to the sub-
micron-range, more precise control of adhesive ligand 
immobilization on a surface down to a few nanometers is 
required to control the geometric exposure of adhesive 
proteins epitopes on a surface. To achieve such spatial res-
olution, block copolymer micelle nanolithography 
(BCML) has been proved to allow for the nanoscale posi-
tioning of ECM ligand molecules (Figure 1). This methods 
imobilizes 4 to 15 nm-sized nanoparticles arranged in a 
quasi-hexagonal pattern, with a adjustable inter-particle 
distance between 20–200 nm [1]. Nanoparticles are then 
used for binding of adhesive ligands (Figure 1). Besides its 
unique ability to precisely position single molecules at na-
nometer length scale, it is suitable for functionalizing large 
areas and can be used with hydrogel polymers such as 
Poly(ethyleneglycol) (PEG).

Objective of this study was to optimize surface properties 
of micro-structured polymer biomaterials that might be fi-
nally used in basic cell biology studies, regenerative medi-
cine or medical implants [13]. Combining soft lithography 
and BCML, we developed PEG-surfaces with a microme-
ter-sized topography, with nanometrical control of adhe-
sive cell ligands distribution. Using these substrates, we 
investigated in a model study the contact guidance behav-
ior of human fibroblasts.

Figure 1. Scheme representing the combination of the 
two methods for micro- and nanofabrication of hierarchi-
cally structured hydrogels.

2 Materials and Methods

Photolithography Photolithography was performed as 
previously described [13], briefly silicon wafers were cov-
ered with positive photosresist (ma-P1210 photoresist Mi-
cro Resist Technology, Germany) by spin-coating (Laurrell 
Technologies Corp., USA). After curing the resist was ex-
posed with UV light through a micropatterned photomask 
(ML & C, Jena, Germany) using a mask aligner MJB4 
(Süss Microtech, Germany). The line pattern with widths 
of 2, 3, 4, 5, 8 and 10 µm are arranged on the mask in

10x10mm squares. After developing processes, SiO₂ was 
deposited onto the micro-structured wafer by physical va-
por deposition yielding in the desired micro-topography.

Block copolymer micelle nano lithography (BCML) 
The basic principle of this method is described in [14] and 
illustrated in Figure 1. A 5mg/ml O-xylene (Merck Schu-
charrdt OHG, Germany) concentrated micelle solution of 
the block copolymer PS (1056)-b-P2VP(495) (Polymer 
Source Inc. Dorval, Canada) was created, 35mg of gold 
(III) chloride acid (HAuCl₄ • 3H₂O) (ABCR GmbH & 
Co.KG, Germany) were added, and stirred for 24h. Before 
use, the micelle solution was purified by using a 0.2-
micron membrane filter (Roth). To nanopattern micro-
structured wafers with the block copolymer solution, two 
methods were tested: dipping and spin coating. In the first 
method, wafers were dipped at a defined speed in the block 
copolymer solution. In the second method, wafers were 
spin coated (Npp/A2/AR2, Laurell Technologies Corp., 
USA), for one minute, with 20 µl of the polymer solution. 
Once monolayer of micelles were loaded onto wafers, thes-
t were treated for 45 minutes in a reactive ion etcher 
(Microwave plasma system PS 210 PVATePla America, Inc., 
USA) with W10 gas (10% Argon and 90% Hydrogen) at a 
power of 200 W and a pressure of 0.1 mbar.

Soft lithography By means of soft lithography, nanopat-
tered microstructures were transferred on PEG-DA hy-
drogels (PEG-DA 700, Sigma-Aldrich, USA). First, wafers 
containing both micro- and nanopattern were incubated 1h 
with a linker solution (N-bis (acryloyl)cytastamine) (Alfa 
Aesar, Germany) and protected from light, in order to co-
valently bind gold nanoparticles to PEG. After, 1 mL pre-
synthesized PEG-DA 700 was mixed with 1 mL of dd 
H₂O. The mixture was stirred under a nitrogen blanket un-
til it was transparent. Then 65 µl of the initiator, 4 - (2-
hydroxyethoxy) phenyl-(2-propyl) ketone (Sigma-
Aldrich, USA) was added and stirred for 10 min under a 
nitrogen blanket protected from the light. For hydrogel 
casting, functionalized wafers were covered with a quartz 
glass 3/₄, with the polymer mixture in between. Polymeriza-
tion was achieved under a UV lamp for 10 minutes at λ = 
365nm. Finally, slides with PEG were immersed in dd-
H₂O and stored in a fridge for 48 hours. Wafers and PEG-
gels were characterized by scanning electron microscopy 
(SEM)(Zeiss Ultra).

RGD functionalization and preparation of the PEG-DA 
hydrogels Nano-micro-structured hydrogels were steri-
lized with 70% ethanol for 15min. After that, they were 
rinsed 3 times with PBS, 10min each. Then, hydrogels 
were incubated for 90 min at room temperature, with a 25 
µM RGD solution (RGD-peptide sequence: cRGD T3, 
Peptide Specialty Laboratorie GmbH, Germany), followed 
by 3 rinses of PBS, 10min each. Subsequently, hydrogels 
were washed for 30 min in PBS. Before seeding cells, sub-
strates were rinsed for 2 hours in the appropriate cell me-
dium.
Cell culture  
Human fibroblasts cells from bone were cultured in D-MEM cell media (Gibco) supplemented with 10% fetal bovine serum and 1% antibiotic. The culture medium was changed every second day. Cells were seeded onto hydrogels at a cell density of 50/mm². After 24h, micrographs from cells on substrates were taken with an Observer Z1 inverted light microscope (Carl Zeiss, Germany).

Data analysis  
Microscope pictures were analyzed with ImageJ. The contours of at least 30 isolated cells per condition, were marked manually. The mean cell orientation was quantified through the non-polar order parameter \( S = \langle \cos (2\phi) \rangle \). For \( S = 1 \) cells are oriented perpendicular to the microstructures direction, for \( S = 0 \) cells are randomly oriented, and for \( S = 1 \) cells are totally parallel to the microstructures [15].

![Figure 2](image_url)  
(a) SEM images of micro-structured silicon wafer with a groove width of 4 microns. (b) Micro-structured silicon wafers with gold nanoparticles, the arrows indicate the influence of the capillary effect. (c, d) Gold nanoparticles on PEG-DA hydrogels at two different magnifications (SEM images).

3 Results  

Nanopattern on microstructures  
In order to obtain a homogeneously and precise distribution of gold nanoparticles on the micro-structured silicon wafer two different methods were used. In one method, micro-structured wafers were dipped into the micelle solution at various speeds. Orientation of micro-channels was parallel to the dipping direction. In the other method, the microstructured wafers were modified by spin coating the micelle solution at different rpm. To determine the best conditions for achieving a precise hexagonal immobilization of the gold nanoparticles on the wafers, the resulting pattern were quantitatively evaluated by analyzing SEM images such as in Figure 2 and calculating the mean distance between particles and standard error. In all conditions and with all microstructure groove sizes, a more homogeneous Nano pattern, indicated by smaller standard errors, was found on spin-coated samples than on samples prepared by the dipping process. To optimize the spin coating process and adapting the method to different microstructure sizes, further experiments were performed with variation in spin-coating speed and other parameters. SEM micrographs of wafers with 200 and 350 nm deep microgrooves (2 and 5 \( \mu \)m width) were quantitatively analyzed by measuring the nanoparticles hexagonality and the width of regions with decreased or increased particle density along the edges of the grooves (Figure 2b). Locally varied capillary forces provoked this formation of areas with different nanoparticle densities in comparison with central regions of grooves and ridges. Further optimization of the protocol led to reduced widths of these inhomogeneous regions. At a spin-coating speed of 9000 rpm, the width of these areas was between 340-380 nm (for 200 nm groove height, and 2 \( \mu \)m groove width), and between 515 and 535 nm on wafers with 5 \( \mu \)m wide grooves. On wafers with 350 nm deep grooves, the width of the areas with altered particle density was typically increased by \( \sim \)100-150 nm. The conditions, at which the Au-nanoparticles are arranged with highest hexagonality was also obtained with a spinning velocity of 9000 rpm on wafers with 200 nm deep microgrooves and 2 and 5 \( \mu \)m groove width. In all cases, hexagonality was always highest in the central part of the microstructure and reduced along the edges of the grooves. Average nanoparticles distance was \( \sim 95 \pm 16 \) nm for both wafers with 200 nm deep grooves (2 and 5 \( \mu \)m width).

Nanopatterned microstructure transfer to PEG hydrogels and cell test  
Gold nanoparticles on microstructured silicon wafers were successfully transferred to PEG-DA 700 hydrogels (Figure 2). Both, the microstructure and the pattern with nanoparticles were preserved. The order of the nanoparticle arrangement was not drastically changed and is similarly ordered as on the master wafer. To promote integrin-mediated cell adhesion to the non-adhesive PEG-surface, covalent functionalization of the particles with RGD ligands was carried out. Human fibroblasts were used to test the new micro-nanostructured surfaces.

![Figure 3](image_url)  
Phase contrast microscopy images of human fibroblasts on a PEG-DA hydrogel with 200 nm deep microgrooves and a groove width and separation of 2 \( \mu \)m (a) and 10 \( \mu \)m (b). The Au-nanoparticles are not visible at this resolution.
For these test experiments, PEG-gels with 200 nm deep microgrooves and separation width of 2x2 µm and 10x10 µm were used. As shown in Figure 3, fibroblast cells could adhere very well to the otherwise non-adhesive gels. They adapt their morphology and align parallel to the micro-grooves (contact guidance). Cells were better aligned on 2 µm wide grooves (S=0.94±0.02) than on 10 µm wide grooves (S=0.83±0.06). The determined order parameters are significantly higher than the ones found for cells growing micro-structured PDMS substrates with physisorbed fibronectin (S≈0.45 on 10 µm wide grooves, and S≈0.85 on 2 µm grooves, [15]). We suggest that the small areas with high particle density along the groove edges, as discussed above, provide an additional elongated chemical signal for the cells leading to an improved alignment.

4 Conclusions

In this work, we present a combination of methods for micro-structuring of PEG-hydrogels surfaces and decorating the surface with ordered arrays of gold nanoparticles serving as anchor for cell adhesion ligands. Both methods haven been used, the combination provides a technique to obtain surfaces with structures on two length scales - the molecular length scale of nanometers, and micrometer-sized structures in the order of cell sizes. First tests with human fibroblasts indicate an increased contact guidance of the cells than on conventional micro-grooved surfaces. This new hierarchically structured surfaces could provide a new platform to study cell responses to a combination of different surface chemistries, topographies, and stiffness, to eventually control cell behavior.

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5 References

Plasma nanofilms as biocompatible and antibacterial interface for biomaterials
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Introduction
In the development of biocompatible materials for biomedical applications and biosensors the foreign body response is an important issue. The healing of surrounding tissue often interferes with the function of an implanted biomaterial. Events like protein deposition, hemostasis, inflammation, tissue repair, infections and the encapsulation of the functional part of the implant are the main cause of failure of the implanted device.

Methods
In this study biocompatible nanofilms are produced by means of a plasma polymerization process using a low-pressure magnetron-enhanced 15 kHz glow discharge. This process allows the precise control of the film nature and behavior. The resulting hydrocarbon film has a thickness of a few nanometer and keeps therefore the inherent properties of the substrate material. X-ray photoelectron spectroscopy, dynamic contact angle, Fourier transformed spectroscopy and electrical impedance spectroscopy were used to investigate the chemical and physical properties of these nanofilms. Measurements on protein adsorption gave the possibility to tailor the thin films in the needed direction. Especially the tailored secondary structure of adsorbed proteins was used to produce biocompatible interfaces.

Results
The nanofilms were investigated using different surface analytical methods. Also the interaction in contact with different biological sample materials was tested in-vitro. The precise measurement of the adsorbed proteins indicated a native secondary structure of proteins on these surfaces. Different in-vivo sensor dummies which are in contact with blood and soft-tissue were coated by these nanofilms. The explanted sensors were kept free of any encapsulation by this coating.

Conclusion
The first in-vitro results of the adsorption of blood proteins indicated already a very biocompatible character of these nanofilms. The explanted sensors were kept free of any encapsulation by this coating. These groundbreaking coatings can open the door for many new applications in the field of in-vivo sensors but also other biomedical products.
Experiments of Platelet Adhesion with the Stagnation Point Flow

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Abstract

With the ever-increasing clinical application of intra vascular implants like valves, stents, grafts and ventricular assist devices the problem of thrombo-embolism has received new attention. Such a thrombo-embolic adverse event starts with the adhesion of platelets. This is investigated with the stagnation flow chamber. Objective of this paper is to research the stagnation point flow with new methods in order to elucidate questions like: How does a thrombus form, what is the role of shear rate? In the new experiments reported here fresh human blood was used. It was drawn by venipuncture from a healthy donor into 1 mM citrate solution. Flow rates were 20 and 40 ml/h and the blood entered the circular flow chamber through a tube 650 µm in diameter. Height of the flow space was 480 µm. The platelets were dyed with Mepacrine. Before the blood entered the flow chamber a platelet-activating agent was added. This agent was mixed with the blood with the help of a micro mixer. The flow chamber was placed in an inverted microscope and platelets deposited on the bottom plate of the flow chamber. This was recorded with fluorescent video microscopy. With the help of image processing the surface coverage (covered area / total area) was determined and was plotted as a function of time and shear rate at the bottom plate. The shear rate was derived from computations of the blood flow using computational fluid dynamics (CFD). The platelet deposition of nearly 50 experiments was recorded, and of these a selection of 9 experiments were analyzed and described in this paper.

1 Introduction

Like the Hagen-Poiseuille flow in a tube, the stagnation point flow is a well-defined flow, however, it has a more complex structure. Like the tube flow the stagnation point flow is ubiquitous as well in the natural circulation: for every two tube flows there is a stagnation point flow at the bifurcation. This makes it an important model flow to investigate the influence of flow in Virchow´s triad [1] of three entities – flow characteristics, wall properties and blood properties. In this case blood cells – platelets - and their deposition is studied. In the first experiments by Petchek [2] in 1968 ex vivo dog blood was used. He could observe the deposition of platelets and the generation of larger aggregates – the beginning of thrombus formation. Later experiments were performed in vitro and mostly human platelet rich plasma (PRP) was used [3]. Although stagnation point flows are numerous in the natural vascular system this model flow has not been adequately investigated – in a handbook of hemorheology [4] it is not mentioned at all. Not only in the vascular system are there numerous stagnation point flows, they also occur in any implant of the vascular system, be it vessel grafts, stents, heart valves or ventricular assist devices. Wherever parts of an implant are joined, small steps in the wall occur. At all these steps stagnation flow is generated. The steps may be small and unavoidable in fabrication, but usually they are large in relation to the size of a platelet. Thus the stagnation point flow studied here can serve as a model flow. The flow chamber has a Re-number of 8, which is computed with the diameter of the inlet tube and a flow rate of 40 ml/h. Whole blood is used as a fluid. When it comes in contact with glass, the glass is covered with proteins, which make it antithrombogenic and platelets do not deposit, unless they are activated. Activation is achieved by mixing blood with an activating agent. This models the situation of blood flowing over an implant: platelets are activated by previous non-physiologic flow. The deposition phenomena observed with the microscope occurs in an area of 1800 x 1100 µm at the bottom plate and is a function of shear rate, platelet activation, time and wall material of the bottom plate.

2 Method

2.1 Stagnation point flow

A rotational symmetric flow has been chosen. The shear rate and its distribution on the bottom plate is computed with a computational fluid dynamics (CFD) flow solver Fluent (Ansys Inc.). It is computed using a hybrid mesh with 2.8 million cells and a non-Newtonian viscosity model. Figure 1 shows the flow field and the wall shear rate at the bottom plate. The stagnation point is situated in the center of the flow and has by definition a wall shear rate of zero. Radially the shear rate at the bottom reaches a peak and then slopes down. The wall shear rate at each radius is thus defined by two values: the shear rate and its slope, which is the radial gradient. This slope defines whether a perpendicular velocity close to the plate is directed towards the plate or away from it, which means carrying platelets to or away from the bottom plate.

2.2 Flow chamber

For the realization of the flow a flow chamber has been designed. It consists of the main body, fabricated of acrylic glass with the stainless steel inflow tube with a diameter of 650 µm. The height of the chamber is defined by the spacer, which is made of silicone rubber and has thickness of 480 µm. The bottom is formed by the base plate, which is a microscopic cover glass of 180 µm
thickness and dimensions of 24x50 mm. Finally, all is mounted in an aluminum the frame, which holds it all together. Figure 2 shows a cross section.

![Cross-section of the flow chamber](image)

**Figure 1** Streamlines of the radially symmetric stagnation point flow. For clarity streamlines are drawn more densely at the center of the inflow and at the bottom. At the bottom half the shear rate at the bottom plate is plotted as function of radius for a flow of 20 ml/h and 40 ml/h. Most prominent feature is the stagnation point at the center.

### 2.3 Experimental setup

Ahead of each experiment the flow chamber and the tubing is filled with phosphate buffered saline (PBS) solution and all air bubbles are removed. Blood is infused into the system with a Razel R100-EC Syringe Pump from RAZEL® Scientific Instruments and generates the blood flow in the flow chamber. It does so by withdrawing the blood from its reservoir. This method is chosen, because it minimizes the handling of the blood sample. The blood needs to be mixed with an activating agent; otherwise its platelets would not adhere to the glass plate. Adenosindiphosphate (ADP) is used. It is also pumped by a syringe pump, which is the PHD UltraTM Syringe Pump from Harvard Apparatus. The ADP is mixed to the blood in a parallel steam of radius for a flow of 20 ml/h and 40 ml/h. Most prominent feature is the stagnation point at the center.

The fluids are mixed at a flow rate of 1 to 10. Since the blood flow has a low Re-number of 8 mixing does not occur; the fluids would simply flow parallel to one another in a laminar flow, the ADP would form a liquid filament and only activate platelets locally. Therefore, liquids, the blood and the ADP are passed through a laminar flow mixer before they enter the flow chamber. In this laminar flow mixer a parallel steam of blood and ADP A.B (A-ADP, B-blood) is split and re-united in the form A.B.A.B. Next this becomes A.B.A.B. A.B.A.B, another step generates A.B.A.B.A.B. A.B.A.B.A.B. This is repeated six times and this way the interface between the two fluids is doubled each time they come together, finally ending with 128 parallel flow filaments in a 1 mm wide channel. This represents a blood flow with homogeneously mixed activated platelets. Two different flow rates – 20 ml/h and 40 ml/h are used in the experiments.

### 2.4 Treatment of blood

As in Petscheks [2] original experiments, but different to later studies, blood is used. Blood is drawn from a healthy male donor after informed consent. It is drawn into S-Monovette® (Sarstedt AG, Germany) containing a 0.106 M solution of the anticoagulant Citrate (equivalent to 3.1% trisodium citrate). After every donation a blood sample is used for the assessment of a complete blood count, showing the values of various parameters e.g. hemoglobin, erythrocytes, monocytes, thrombocytes, hematocrit in the blood. Upon arrival in the laboratory the blood is mixed with the fluorescent dye Mepacrine so platelets become visible under the fluorescence light. 50 µl Mepacrine for every 1 ml of blood was used. The Mepacrine solution is prepared beforehand using 20 ml of phosphate buffered saline (PBS) solution and 0.01 g of Quinacrine dihydrochloride (of Sigma-Aldrich Chemie GmbH, Germany). 4 – 8 ml of blood is needed for each run of the experiment in order to be able to carry it out in an adequate manner. The ADP used for the experiment is in form of a 2µM solution mixed using dimethylsulfoxid (DMSO) and ADP in powder form from Serva Electrophoresis. The experiments were carried out within 3 hours after the taking the blood sample.

### 2.5 Microscope

A Leitz Fluovert FU inverted microscope is used with a PL FLUOTAR 6.3/0.20 lens. The platelets are made visible with fluorescent. For this purpose the light of a 50 W mercury vapor lamp is passed through a Leica filter block. It contains a band-pass filter, which permits light of frequencies between 355-425 nm to pass, blocking off all others above and below. The dyed platelets light up and fluoresce – emit light of another and higher wavelength. The light is passed again through the filter block and is separated from the excitation wavelength and then passed through the emission filter which lets through all wavelengths above 460 nm and blocks all others. With this procedure platelets, but also leucocytes, become visible,
because they have absorbed the fluorescent Mepacrine. The light is then ready to pass the specialized lens. The camera is connected to the lens with a C-Mount Adapter 0,5 from Leitz.

2.6 Recording

A uEye-Monochrom camera UI-2230-M from Imaging Development Systems GmbH is attached to the microscope. It has a resolution of 1024x768 pixels. A frame rate of 1.3 1/s with an exposure time of 630 ms was used.

2.7 Image processing

The video sequences are recorded in the audio video interleave format (avi) and stored on a laptop computer. The images are further processed with a Matlab program. The images are represented in grey-scale values from 0 (black) – 255 (white). The images are not completely uniform lit and some parts are darker than others. This is taken into account by averaging 10 images pixel wise after focusing and before starting the flow. This background image is then subtracted from every image before further processing. Next a singular platelet image is analyzed with regards to gray value in order to determine a threshold gray value. This has been determined to 70: pixels below are converted to zero – black, and above to 255 – converted to white.

With this threshold all images are binarized. Now platelets appear as white flecks on a black background. Since it is a radially symmetric flow, equal shear rate at each specific radial distance from the stagnation point can be assumed. As observation shows, platelets do not evenly distribute at each radius. A stochastic element in the deposition must be acting; it is assumed that this is the Brownian motion of the platelets. At a short distance from the bottom plate this Brownian motion determines the contact and thus the deposition. In order to evaluate the shear influence on the deposition the deposited platelets – by now transformed into white pixels, – are counted circular ring area wise. This is performed by dividing the number of pixels on a circular ring by its area. The result is an average surface coverage as a function of the radius.

3 Results

![Figure 4 Development of surface coverage as function of radius for a flow of 20 ml/h at different moments - 400, 440, 480 and 520 seconds after start of the pumps.](image)

When the pumps are set in motion at the beginning of an experiment, firstly the PBS is displaced. In the moment the blood enters the chamber, the previously visible inlet opening disappears and a few individual platelets adhere to the glass. This permits focusing of the microscope and recording starts. In this early phase of the experiment flowing platelets are to be seen that do not deposit and only slide past the surface. They appear as fuzzy white streaks on the surface. Bit by bit the platelets then deposit and adhere. Over the course of the experiment a characteristic pattern of deposited platelets develops: little deposition occurs directly at the center – the stagnation point. A ring of high surface coverage around the center and a decrease of surface coverage at larger radii is observed. From single platelets at the beginning larger aggregates develop. As the flow has radial symmetry, also the deposition shows radial symmetry, however with a strong stochastic influence. Figure 3 shows four images from the recording with a flow of 20 ml/min. The images are taken at 400, 440, 520 and 560 seconds after start. Glass was used as the surface material. For the quantification of the experiments the ring wise averaged platelet deposition – the surface coverage – is plotted over the radius. Figure 4 shows the development of surface coverage as function of radius for a flow of 20 ml/h at different moments - 400, 440, 480 and 520 seconds after start of the pumps. If one compares different experiments, different developments are observed. Although the time is recorded from the start of the pumps, the deposition does not appear at the same time steps. This time – the numbers given above – to the onset of deposition varies greatly. The cause for this is still not known exactly. It is assumed, the initial mixing of blood and ADP solution is not well defined and dependent on the manual filling of the syringes. However, setting the start point in time according to the steepest rise of surface coverage can compensate this different onset of the platelet deposition. In figures 5 and 6 this surface coverage is plotted as function of radius for experiments at 20 and 40 ml/h and the average of the experiments.
Objective of these new experiments was to investigate the usefulness of the stagnation point flow model. This goal has been achieved: compared to previous experiments, the reproducibility of results has been greatly improved. This is attributed mainly to two causes: the use of blood of the same donor for all experiments and the skill of the technicians drawing blood. Given the sensitivity of the platelet it can be considered an art to bring the blood unharmed from the vein into the syringe. Professional technicians have been employed, who do nothing but drawing blood all day. Further work will be needed to expand the usefulness of the stagnation point flow model. This concerns the three entities flow, wall and blood. The flow is to be modified to include flows, which produce higher shear rates. This is achieved with higher flow rates, up to 80 ml/h. The wall has up until now been untreated glass, onto which platelets do not deposit, because it is covered with proteins in the first contact of blood. Platelets need to be activated to achieve a deposit. This is a useful model for implants in the cardiovascular system. These are usually made of materials, which resist platelet deposition. Still, the latter is observed because platelets are activated by the implant, either by high shear rates or by partial interaction with the implant material. To investigate such an interaction a variety of materials will be tested. There are a number of blood compatible polymers to be investigated, polyurethane, polyvinylchloride and others. How do they compare? This has not been clarified yet. One oft the materials will be collagen and in this case the platelets in whole blood will be non-activated. This setting models the encounter of platelets with a lesion in a natural vessel, when missing endothelial cells expose collagen of the vessel wall, as it has been experimentally investigated in a simple Couette flow [5]. In regard of the manageability, there is much room for improvement: blood is not used efficiently enough and not fast enough. The whole process of preparing the flow chamber, degassing the tubings and cleaning needs an overhaul. One way to achieve this is to fabricate multiple flow chambers. They could be jointly, degassed, connected to the pumps and in the course of the experiment sequentially perfused. This way the donors blood can be used more efficiently and speedily. The range of the standard deviation is likely to further decrease. Finally, the quantification of Virchow’s Triad is a goal, which has not yet been achieved, although it has been pursued for a long time [1]. This endeavour needs experimental results and numerical models for the interpretation. Due to the scarcity of recent experimental data old data [6] have been used for mathematical modelling and an interpretation [7, 8]. An improved flow chamber and an optimized operational procedure will permit to generate the experimental data required for the development of numerical models.

4 Conclusions

5 References

Characterization and optimization of antibacterial implants in vitro and validation in a small animal model

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Introduction

Biological properties of novel medical implant materials are frequently first characterized in cell culture models. Despite the existence of standard procedures, these assays cannot reflect the complexity of implant-tissue interaction.

Methods

Magnesium as a prospective degradable implant material and magnesium-based Layered Double Hydroxides (LDH) as a potential degradable implant coating that could be used for controlled drug delivery were both investigated in cell culture assays. Proliferation-controlled cells were used to verify the biocompatibility of LDH coatings. To monitor antibacterial effects of the materials, luminescently labelled P. aeruginosa bacteria were employed in combination with an in vivo imaging system. For in vivo testing a mouse model was established using subcutaneous implants that could be infected with the luminescent bacteria.

Results

LDH coatings were highly biocompatible in various mammalian cell culture assays whereas cell proliferation on pure magnesium surfaces was reduced. In comparison, in the mouse model, both materials were highly compatible with soft tissue. In vitro antibacterial efficacy could be achieved with both, pure magnesium and with antibiotic-releasing coatings. However, in vivo the antibiotic effects differed considerably from the cell culture results.

Conclusion

Cell culture tests could be used to identify and optimize specific biological and mechanical characteristics of the materials investigated but they could not accurately predict all aspects of the in vivo performance. After optimization, LDH coatings showed highly promising properties as local drug delivery system in vivo.
In vitro degradation and biomechanical testing of magnesium alloys

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Introduction

Implants made of magnesium alloys are promising for orthopaedic applications, because (i) they are biodegradable and eliminate the need for follow-up surgery to remove metal parts; (ii) their mechanical properties are similar to those of cortical bone, which may minimize inhomogeneous stress transfer (‘stress shielding’); and (iii) they have favourable osteoconductive properties.

Methods

In vitro studies were applied to investigate the degradation behaviour and cytotoxicity of magnesium alloys, as well as the biomechanical strength of manufactured implants. The degradation rate was evaluated by the weight loss method after immersion in Hank’s Balanced Salt Solution (HBSS). A cytotoxicity test according to EN ISO 10993-5/12 was conducted with L929 cells to evaluate potential toxic leaching, and ICP-OES was applied to quantify the Mg ions (Mg²⁺) released from the material. Intramedullary nails made of LAE442 were loaded for 500,000 cycles in HBSS and distilled water to determine the fatigue resistance.

Results

The tested magnesium alloys had degradation rates of 2.89 mm/y (MgCa0.8), 0.63 mm/y (ZEK100), and 0.24 mm/y (LAE442) in HBSS. The results of the first two alloys were verified via the determination of the released Mg²⁺ from the alloy, which showed a slower Mg²⁺ release rate for the ZEK100 alloy. The magnesium alloy Mg-2La showed no toxic effect on L929 cells, the 100%-extract of LAE442 led to a minor reduction of the relative metabolic activity. Promising fatigue strength was only observed with LAE442 nails in distilled water.

Conclusion

In vitro tests provide a variety of different investigation opportunities under standardized conditions. The degradation rate differs depending on the type of magnesium alloy and the test parameters. The premature loss of function of degradable implant materials is undesirable for orthopaedic applications.
Nanogels for Zinc Oxide Nanoparticles Encapsulation and Their Utilization for Wound Therapy

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1 Introduction

Traces of heavy metals are of great importance for human bodies. Zinc for example is essential for over 300 different enzymes. After thermal injuries, fluid will be lost by abandoning the wound consequenting dramatic loss of trace elements such as, copper, zinc and selenium. These elements are not only essential of vital functions but also for wound healing process, especially in case of thermal injury. The method introduced here is aiming on wound-pad preparation by coupling clean nanoparticle preparation, encapsulation and electrospinning. Therefore, laser ablation and in situ nanogel polymerization are performed and characterized regarding the release of metal ions essential for wound therapy.

2 Methods

Preparation of PVCL-PEG Nanogels is done in a batch chamber and stirred mechanically. VCL, PEG-MA and the crosslinker (BIS) are given into the chamber. After annealing up to 70 °C, the initiator is added and the reaction left stirring for 2 hours. The products are freeze-dried without further purification.

Electrospinning is done by mixing nanogels and PCL in a Methanol-Toluene mixture (1:1) at PCL concentration of 12 wt.-%. Electrospinning is applied by an acceleration voltage of 20-25 kV, a flow rate of 0.5 mL/h and target to spinneret distance of 15-20 cm.

3 Results

Here, we present a facile approach of nanogel design, suitable for laser ablation due to their small size resulting in transparent dispersion. The non-turbidity is mandatory for the laser process, so we introduce an approach of nanogel synthesis in situ which is suitable in order to conjugate to the metal nanoparticles received by ablation. Afterwards, the encapsulated nanoparticles (ZnO) were applied by electrospinning and characterized regarding their zinc ion release properties.

4 Conclusion

The introduced method of nanoparticle preparation by Laser deposition and the in situ preparation of nanogels in order to receive encapsulated composites has been shown to work very effectively like methods shown before. The fibers show zinc ion release profiles which can be easily adjusted by ZnO content in the fiber-pads.

4 References


Image 1: Plan of the preparation of wound healing supporting fibers with laser-generated nanoparticles.
Improving the antibacterial effect of polyamide 12 by functionalization of titanium dioxide nanoparticles

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Introduction

Microorganisms in hospitals are becoming an increasing problem. The rate of infection has risen due to surface contaminations with those. To prevent infections, titanium dioxide (TiO$_2$) is used in surfaces to destroy microorganisms and pollutants and by that sterilizing it. Nanoparticles enhance this effect, due to that an allover distribution inside of the polymer is aimed.

Methods

TiO$_2$ nanoparticles are functionalized with styrene maleic anhydride (SMA) in order to improve the dispersion within a polyamide 12 (PA12) matrix. The structure of the functionalized TiO$_2$ is characterized by Fourier Transform Infrared Spectroscopy (FT-IR), physisorption (BET) and Scanning Electron Microscopy (SEM). Two composites are compounded, PA12 with 10 wt% functionalized TiO$_2$ and PA12 with 10 wt% pure TiO$_2$. Those composites are characterized by SEM, Total Organic Carbon (TOC) method and contact angle measurements. The antibacterial properties of the composites is investigated by decomposing experiments with rhodamine B.

Results

The results show that the TiO$_2$ nanoparticles can be successfully functionalized with SMA. This is confirmed by a decrease of the particle surface, measured with BET, and the building of small plates and no single nanoparticles, visible in the SEM. The decomposition experiments with rhodamine B showed that the composite with the functionalized TiO$_2$ exhibited a higher decomposition rate compared to the other one. This can be interpreted as a higher disinfectant effect.

Conclusion

All these investigations shows that the functionalizing of TiO$_2$ with SMA increases the antibacterial effect of PA12. The act of disinfectant is higher for the PA12 with functionalized TiO$_2$, compared to the one with regular TiO$_2$. To determine the distribution and improve the parameters, further research is necessary.
Polymeric Hard-Soft-Combinations by Multi-Component Injection Moulding for Small Medical Devices

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Introduction

The combination of hard and soft polymeric materials via multi-component injection moulding plays an important role in the manufacturing of plastic devices for medical applications. Especially small multi-component parts integrating different functionalities (e.g. damping, sealing, haptic) are a challenge for good adhesion and safe products. Examples therefore are small housing parts of hearing aids or small pumps for the exact dosing of medicaments. The aim of this study is to achieve good adhesion of a soft elastomer on different thermoplastic substrates tested in small geometries. Thereby, liquid silicone rubber (LSR) and thermoplastic elastomers (TPE) are compared directly to each other.

Methods

The adhesion of different TPE and LSR of low shore hardness (40A) is analysed on selected thermoplastic substrates. Our focus is on medical grade materials manufactured by injection moulding and tested in a miniaturized peel test specimen, as medical parts are often of small size. In order to improve adhesion of LSR on the thermoplastic surfaces, an open air plasma treatment is conducted. For analysing the adhesion, a 90° peel test is conducted; fractured surfaces are examined by optical microscopy and scanning electron microscopy.

Results

The analysed TPE-S types show good adhesion results on polypropylene (PP) and polyethylene (PE) substrates. However, a quantitative evaluation of the peel test is difficult due to the low strength of the TPE. The adhesion modified LSR types show adhesion to polybutylene terephthalate (PBT) and polyamide 12 (PA12). After a plasma treatment, adhesion is improved and LSR offers good adhesion to various substrates (PE, PP, PC, PPS, PEEK).

Conclusion

For small geometries, often the mechanical strength of an elastomer is the critical factor for failure – not the adhesion strength of the composite. In further studies, the influence of a miniaturized test geometry and medical fluids or sterilization processes should be analysed in detail.
Influence of Sol-Gel coatings on the corrosion behaviour of MgZn1 foams with 30 % porosity

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Abstract

The aim of this study was to investigate the ion release behaviour of MgZn1 foam with 30 % porosity and three different sol-gel coatings by static immersion test. Compared to uncoated samples the corrosion could be reduced significantly with all three coatings. Under these test conditions Coating 1 revealed the best corrosion protection.

1 Introduction

Open porous Mg foams can be considered as candidate material for bioresorbable implants [1]. To adjust the corrosion rate of Mg foams coatings are an obvious option. Sol-gel coatings show excellent chemical stability, oxidation control and enhanced corrosion resistance on metal substrates [2]. Furthermore, considerable research have been made in the past few decades in the field of bioapplication of sol-gel materials. These materials are known to be highly biocompatible thereby making them attractive candidates for medical devices such as coatings for implants [3].

The aim of this study was to investigate the ion release behaviour of MgZn1 foam with 30 % porosity and three different sol-gel coatings by static immersion test.

2 Materials & Methods

2.1 Manufacturing of the Mg-foams

Open porous magnesium materials were manufactured using the so called place holder method which is schematically displayed in Image 1. For this 25 vol.% of a granulated place holder substance (ammonium hydrogen carbonate, NH₄HCO₃, Alpha Aesar, particle size 355-1000 μm) was added to a powder mixture consisting of 99 wt.% magnesium powder (Mg 99.8, Ecka, particle size 100-315 μm) and 1 % zink powder (Zn 99.995, Ecka, particle size < 315 μm). Portions of 1 g of this powder mixture were consolidated by uniaxial compaction in a cylindrical die of 15 mm diameter using a compaction force of 100 kN to obtain cylindrical tablets of about 3.5 mm thickness. In a final step the compacted samples were sintered in an argon atmosphere using a slow heating rate of 1 K/min to allow the place holder to volatilise. The holding time was 2 hours at a temperature of 605 °C. During this sintering step a strong metallic bonding between the metal powder particles is generated by diffusion processes, so that stable specimens are obtained which can tolerate high mechanical loads.

2.2 Application of sol-gel coating

Three different sol-gel coatings were selected from available stock and applied on the porous MgZn1 foam discs. The coatings were selected by matching technical requirements of this study, such as good adhesion to the metal substrates and low viscosity, which enables the coating to penetrate into a porous structure.

The main components of the sol-gel coatings are organosilanes, also known as Ormosil, organically modified silica. Generally, by including organic modified silane precursors in the coating formulation, it is possible to modify the physical, chemical and mechanical properties of the coated object. The selected coatings and characteristic are:

- Coating 1, a sol-gel coating consisting primarily of a SiO₂ network. Cured at 140 °C, 1 hour.
- Coating 2, a porous sol-gel coating which erodes at a slow pace in contact with water. Cured at room temperature (RT), 2 hours.
- Coating 3, a sol-gel coating with good anti-corrosive properties. Cured at 200 °C, 1 hour.

The sol-gel coatings were applied by a dipping procedure (Image 2). Discs of the porous MgZn1 foams were placed in a container which was then filled with the coating so the discs were completely covered. After approximately 5 min. the MgZn1 discs were collected and excess coating was removed from the discs by run-off and the backside was wiped clean. The discs were placed on a flat surface for a 5 min solvent flash-off. For Coating 1 and 3 the sol-gel coating was cured at elevated temperature in an oven. Subsequently, the backside was coated by placing 3-5 drops of
the coating and brushing this into an even layer. The MgZn1 foams were cured again providing the finished coated foam.

From all electrolytes the pH-value was measured (pH-meter 629, Metrohm, Ostfildern) and the results were graphically presented for each coating depending on immersion time.

3 Results

The densities of the samples were determined by measuring the weight and the geometric dimensions of each specimen. Density values were found to lie in the range between 1.18 and 1.34 g/cm³, corresponding to porosity values between 25 and 34 %. The mean values for density and porosity were 1.26 g/cm³ and 30 %, respectively. In view of the place holder content of only 25 % this indicates that some additional porosity is present in the samples. This is confirmed by metallographic sections as shown in Image 4 where besides the bigger pores resulting from the place holder particles also some smaller pores can be observed. The smaller pores are situated at interparticle boundaries and can thus be attributed to the powder metallurgical manufacturing route.

The penetration of coating into the porous MgZn1 foam were analysed by dipping MgZn1 foams 5 min in a flouresecent variant of Coating 3. The viscosity of this flouresecent variant of Coating 3 is similar to the viscosity of the three selected coatings. After curing, the cross section of the MgZn1 foam was investigated by UV-light which shows that the coating penetrates into the MgZn1 disc as seen in Image 5. This study shows that the coating not only provides a protective layer on the outside of the MgZn1 foam but also protects the cavities inside the structure.
Fluorescent inspection of the cross section of a coated MgZn1 disc. The used fluorescent coating is a variant of Coating 3.

Release of Mg and Zn ions and pH alteration as a function of immersion time is shown in Images 6, 7 and 8.

In the uncoated foam after 6 h the mean Mg release increased significantly from 30 µg/cm² to a value of 3880 ± 920 µg/cm² after 24 h. A similar effect was found in Zn, in which case the ion release reached a value of 5.4 ± 0.8 µg/cm² after 24 h. Beyond 24 h no further analysis could be done, because the samples began to fall apart.

In contrast, with all three coatings the corrosion rate of the MgZn1 foam was reduced. MgZn1 foams with Coating 1 and 2 showed after 96 h immersion precipitations in the solutions, therefore, chemical analysis were not continued. Coating 3 was stable up to 216 h without precipitations.

Image 5 Flourescent inspection of the cross section of a coated MgZn1 disc. The used fluorescent coating is a variant of Coating 3.

Image 6 Mg-release of the coated and uncoated samples

Image 7 Zn-release of the coated and uncoated samples

Image 8 Alteration of the pH value with immersion time for each coating.
Between 12 and 24 h the pH-value increased from 7.4 to 11 and levelled off between pH 11 and 12 until the end of the immersion time (Image 8). Image 9 represents the accumulated mean Mg plus Zn ion release. The data clearly demonstrate that with all three sol-gel coatings the chemical solubility of MgZn1 foams can be significantly reduced. Because of the precipitates and thus a limited chemical analysis a ranking of the coatings could be carried out only after 96 h. Taking into account the scatter in the data, Coating 1 and Coating 2 were similar, with slightly better values in Coating 1. During the initial time Coating 3 was poorer by about a factor of 3. It can also be seen that the corrosion protection by the coating wears off after about 96 hours. Compared to uncoated samples with a Mg release of 30 µg/cm² after 6 h and 3880 µg/cm² after 24 h the corrosion could be reduced significantly with all three coatings. Under these test conditions Coating 1 revealed the best corrosion protection during the first 96 h of immersion.

4 Conclusion

In contrast to uncoated MgZn1 foam, all coated samples were stable up to 96 h. MgZn1 with Coating 3 was durable up to 216 h, while with Coating 1 and 2 after 96 h deposits were formed in the solutions, which prevented a proper analysis. After 96 h the mean Mg release was 69 (Coating 1), 127 (Coating 2) and 319 (Coating 3) µg/cm² and, Zn release was 0.21 (Coating 1), 0.42 (Coating 2) and 0.82 (Coating 3) µg/cm². Between 12 and 24 h the pH-value increased from 7.4 to 11 and remained on this level until the end of immersion time. Compared to uncoated samples with a Mg release of 30 µg/cm² after 6 h and 3880 µg/cm² after 24 h the corrosion could be reduced significantly with all three coatings. Under these test conditions Coating 1 revealed the best corrosion protection during the first 96 h of immersion.

5 References

Quantitative analysis of cartilage surface by confocal laser scanning microscopy

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Introduction

Articular cartilage plays a crucial role in the tribology of joint movement. Detailed surface roughness analysis of cartilage is important for the diagnosis and treatment of osteoarthritic articular cartilage. The roughness of cartilage has been already determined by various methods like atomic force microscopy (AFM), scanning electron microscopy (SEM), profilometry and confocal laser scanning microscopy (CLSM) in multiple studies. Due to our knowledge the use of CLSM for quantitative roughness measurements of cartilage morphology has not been reported.

Methods

The samples consisting of cartilaginous tissue and subchondral bone tissue were prepared from porcine tibia plateau. Some samples were treated with commercially available emery paper (P180, P120, P60). A well-defined grinding procedure was applied to the specimens. The specimens were fixed, stained and lyophilized. The control specimens were light-microscopically assessed as having smooth, the sanded specimens as having intermediate, rough and very rough surfaces, respectively.

For quantitative characterisation of the surfaces various roughness parameters were measured using Olympus LEXT OLS 4000 CLSM. Repeated measurements at different locations on each sample surface were undertaken to establish statistical inference. The region of interest of each image was set to 1281 µm x 1279 µm with the magnification 216x. Qualitative images of the surfaces were also acquired.

Results

A correlation was found between the surface roughness of the sanded cartilage and the grit size of the emery paper.
The results indicate a dependency between the roughness values and the sample preparation as well as the setup of the microscope. For comparison it is important to use the same objective lens, tilt corrections and to apply filters to separate surface form, waviness and roughness information.

Conclusion

The new method should be sensitive to differences as small as 1 µm in mean roughness and may provide a reproducible process of quantifying the cartilage changes in osteoarthritis.
In vitro human capsulorhexis specimen viability test for assessing biocompatibility

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Abstract

In routine cataract surgery the opacified lens can be replaced without restoring accommodation. Using an injectable lens into the sustained capsular bag shall restore accommodation. For assessing biocompatibility of newly developed lens polymers, appropriate cell viability test systems have to be developed. Here, we performed a test utilizing capsulorhexis specimens as material, to which the fluorescent CellQuanti-Blue viability assay was applied and compared to the previously used Live/dead viability assay. Both tests correlated positively, and revealed stable cell viability in capsulorhexis specimens cultivated for up to 48 h. The reliable detection of mitomycin C cytotoxicity to the specimens verified the applicability of the system to test the biocompatibility of different materials, including newly developed lens polymers.

1 Introduction

Cataract is a visible opacity in the lens substance, caused by post-translational or conformational changes in the lens proteins, loss of reduced glutathione as antioxidant and loss of free-radical scavenging capacity [1]. Nearly half of all cases of blindness are caused by cataract, the most common cause of reversible blindness worldwide [2]. Treatment essentially involves phacoemulsification of the opacified lens and its replacement by artificial intraocular lenses (IOL). The major disadvantage of currently used IOLs is that they are not capable to change shape and position like the natural crystalline lens and, consequently, to achieve accommodation. One approach in research focuses on development of injectable lenses, which are the most promising concept for restoring lost accommodation [3-5]. Prerequisites for an injectable IOL material are an appropriate refractive index and biocompatibility. While monolayer cell culture systems may be of value in testing biocompatibility, they do not completely mimic the natural environment of a crystalline lens.

Hence, the goal of the present study was to test the suitability of the fluorescent CellQuanti-Blue viability assay for human capsulorhexis specimens containing lens epithelial cells on their natural substrate as adequate target cells for biocompatibility studies.

2 Methods

2.1 Material

Human anterior capsulorhexis specimens were received from the Department of Ophthalmology at the Rostock University Medical Center. During standard cataract surgery circular specimens with a diameter of about 3 mm were obtained from patients aged 36–94 years.

2.2 Culture conditions

Immediately after resection, the specimens were placed in Dulbecco’s modified Eagle’s medium (DMEM) with 4.5 g/l D(+)-glucose, 4 mmol/L L-glutamine, and 1 mmol/L sodium pyruvate (PAA, Cölbe, Germany), supplemented with 44 mmol/L NaHCO₃ (Merck, Darmstadt, Germany), 5 mg/ml gentamicin (Sigma-Aldrich, Steinheim, Germany), 2.5 mg/ml amphotericin B (PAA), 10% FCS (PAA), and 20 mmol/L HEPES (PAA) to allow for pH stability before arriving in the laboratory. Then, specimens were transferred into 24-well microtiter plates containing DMEM without HEPES, adjusted to pH 7.4. Specimens were cultured, incubated with MitC, and the CellQuanti-Blue viability assay performed under standard conditions at 37 °C, 5% CO₂ and relative humidity of 95% in an incubator (Heraeus BB 6220 CU O₂ incubator, Fisher Scientific, Schwerte, Germany).

2.3 Assessing viability

For evaluation of lens epithelial cell viability capsulorhexis specimens were cultured for up to five days, and viability tests were performed at certain time points (24, 48 and 120 h).

2.3.1 CellQuanti-Blue assay

For a quantitative analysis of cell viability DMEM was removed and replaced by a 1:10 dilution of CellQuant-Blue™ Cell Viability Assay reagent (BioAssay Systems, Hayward, CA, USA) in DMEM. Thereafter specimens were further incubated for 2 h. Subsequently, fluorescence of the supernatant was measured (544 nm excitation, 590 nm emission) (Fluostar Optima, BMG, Offenburg, Germany).
2.3.2 Live/dead assay

Additionally, lens epithelial cell viability was evaluated using a two-color fluorescence assay (Live/dead™ assay, Invitrogen, Paisley, UK), which allows for a simultaneous determination of living and dead cells and has been established previously in our group [6]. Calcein AM and ethidium homodimer-1 were used to stain viable and dead cells, respectively. Finally, all cell nuclei were stained using Hoechst 33342 (Sigma-Aldrich, Steinheim, Germany). Specimens were photographed under a confocal laser scanning microscope (FluoView FW1000, Olympus, Hamburg, Germany) with 2.5× and 10× objectives (Image 1a and b, respectively). Stained cell nuclei areas were extracted from the photos (Paint.NET v3.5.4) (Image 1c) and their numbers quantified (CorelDRAW X6).

![Image 1](https://via.placeholder.com/150)

**Image 1** a) and b) triple fluorescence of a specimen, c) extracted total (blue) and dead cell nuclei (red).

2.4 Testing mitomycin C cytotoxicity

For testing cytotoxicity, a deep-frozen stock solution of MitC (Sigma-Aldrich, Steinheim, Germany) (1×10⁻² mol/L in ultrapure water) was used. Concentrations of 1×10⁻⁶ and 1×10⁻⁴ mol/L MitC in DMEM were added to the specimens. As negative controls (NC) specimens were cultured in DMEM only. Cell viability before and after 48 h of drug incubation was determined as described above (2.3.1).

2.5 Data analysis and presentation

Means and standard deviations (SD) were calculated (Microsoft Office Excel 2007) and presented by column or scatter plot diagrams (GraphPad Prim 5).

The number of living cells per square millimeter was calculated as the difference between total cell number and dead cell number, divided by the area photographed.

Statistical analysis was performed by repeated measures ANOVA [7] with Bonferroni’s multiple comparison post hoc test [8] (GraphPad Prim 5).

Both viability tests were tested for correlation by calculating Pearson’s correlation coefficient (GraphPad Prim 5).

3 Results

In the CellQuanti-Blue assay, the capsulorhexis specimens revealed a good viability for up to two days after resection as shown by fluorescent units. Cell viability was, however, gradually diminishing with increasing incubation time. Compared to one-day cultivation, after five days samples yielded a 28 % decline in viability, and this difference was statistically significant (p < 0.05) (Image 2).

![Image 2](https://via.placeholder.com/150)

**Image 2** Cell viability of human capsulorhexis specimens in culture medium (means ± SD).

The Live/dead assay revealed comparable findings, and a good correlation could be shown between the two approaches. Image 3 demonstrates exemplarily how the number of viable cells after 120 h-incubation correlates between both tests. A Pearson’s correlation coefficient with a value of +1 denotes a completely linear positive correlation between two parameters investigated. Here, we calculated a significant correlation of +0.81 (p = 0.0009) between both test methods.

![Image 3](https://via.placeholder.com/150)

**Image 3** Linear positive correlation between both viability assays.

To verify the applicability of the fluorescent CellQuanti-Blue viability assay to capsulorhexis specimens for future biocompatibility studies, we incubated the samples with MitC, whose cytotoxic effects were predictable. The samples were incubated with two different concentrations of MitC for 48 hours, followed by viability measurement. As shown in image 4, a significant reduction in cell viability was observed after application of both (10⁻⁶ and 10⁻⁴ mol/L) concentrations of MitC, compared to pre-incubation values (p < 0.05 and p < 0.01, respectively). MitC revealed a cytotoxic effect with a viability decrease of 48 % and 53 % at 10⁻⁶ and 10⁻⁴ mol/L, respectively. On the other hand, the untreated specimens (NC) showed a
slight increase in viability, which, however, was not statistically significant (Image 4).

\[ \text{Cell viability (fluorescent units)} \]

\begin{align*}
0 \text{h} & \quad \text{NC} & 48 \text{h} & \quad \text{NC} \\
0 \times 10^{-6} \text{ mol/L MitC} & \quad \text{p<0.05} & 4 \times 10^{-4} \text{ mol/L MitC} & \quad \text{p<0.01}
\end{align*}

Image 4: \(10^{-6}\) and \(10^{-4}\) mol/L MitC reduce viability, NC: control samples without MitC.

### 4 Conclusion

In the present study we tested the suitability of the fluorescent CellQuanti-Blue viability assay for human capsulorhexis specimens as an appropriate in vitro biocompatibility test system. The advantage of capsulorhexis specimens over lens epithelial cell monolayer cultures is that lens epithelial cells in the former reside on their natural substrate, the basal membrane, better mimicking the in vivo situation.

Using the Live/dead assay, Sternberg et al. showed a lens epithelial cell density of \(1.361 \pm 482\) cells/mm\(^2\) of untreated capsulorhexis specimens measured within 2 h after resection [6]. Our data (880,48 \(\pm\) 744,46 cells/mm\(^2\)) from a five-day cultivation correspond to 65% of the value found by Sternberg et al., showing a decline in cell viability by 35%. This is similar to what we measured using the fluorescent CellQuanti-Blue assay, where a decline in cell viability by 28% was calculated between one-day and five-day cultivation.

Assessing human lens epithelial cell viability, the CellQuanti-Blue assay applied to human anterior capsulorhexis specimens showed comparable results to the Live/dead assay, as ascertained by a good correlation between both data sets. Cytotoxic effects of MitC were reliably detected. Thus, an appropriate in vitro test system with adequate target cells for biocompatibility studies was established and can be used for testing newly developed lens refilling materials.

### Acknowledgement

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### References


Development of a Biocompatible Lubricous Coating for Biomedical Applications

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Keywords: implant coating, hydrogels, hyaluronic acid, layer-by-layer deposition

Abstract

A biocompatible lubricous surface coating based on poly(L-lysine) or L-lysine and hyaluronic acid multilayers was investigated. No chemical modification of polyelectrolytes was necessary and the final architecture was obtained using the layer-by-layer deposition method. Investigation of coating morphology and coating thickness show that all tested coating compositions result in a very smooth and homogenous coating with thicknesses between 0.6 and 2 µm.

1 Introduction

Water soluble, biocompatible compounds that impart lubricity to the surface of otherwise non-lubricious materials are desirable for biomedical devices which are inserted or implanted into the body. Such medical devices may include cochlea implants, pacemaker electrodes, urethral catheters or coronary catheters which are utilized to deliver a stent, stent-graft, balloon catheters or other expandable medical devices etc. Especially hydrophilic lubricious coatings are interesting in order to overcome limitations using hydrophobic coatings such as silicone. Hydrophilic coatings have been known to be smooth, run off, lose initial lubricity rapidly and for lack abrasion resistance, when exposed to an aqueous environment. Residual amounts of silicone have also been known to cause tissue reaction and irritation in patients [1]. The loss of lubricity can lead to discomfort during insertion or removal of the device.

Hydrogels, which swell in an aqueous environment, are capable of manifesting lubricity under wet conditions and are currently already utilized as lubricious coatings for medical devices. When hydrated, these substances have low frictional forces in humid or wet and are therefore suitable for medical devices. When hydrated, these substances have low frictional forces in humoral fluids including saliva, digestive fluids and blood, as well as in saline solution and water. Such substances include poly(ether-ester) [2], poly(vinylpyrrolidone) [3] and polysaccharides, such as hyaluronic acid (HA).

Biocompatibility is a general requirement, especially for implants with long residence time in the body, since the coating remains in long-term contact with the biological system, but also for implants with only a short contact time with the human body. Especially during insertion or implantation of the medical device, the coating can shear off due to high frictional forces and particles would remain in the organism e.g. in the arterial pathway. Within this context, we investigated the deposition of HA coatings to polymer surfaces as model implant application to reduce the coefficient of friction of the implant surface. HA is a biodegradable, biocompatible, nontoxic, nonimmunogenic and noninflammatory linear polysaccharide present in the extracellular matrix of all higher animals. In covalently crosslinked condition, HA forms a hydrophilic polymer network, which may absorb a multiple of its dry weight in water. Nevertheless, crosslinked HA-particles are no longer water-soluble. For this reason we aim at a surface coating based on non-crosslinked HA. This lubricious property, combined with its biocompatibility, has led to different medical applications of HA, e.g. in ophthalmic surgery to protect delicate eye tissues during surgical manipulation [4], in orthopedics to replace synovial fluid in painful arthritis [5], and in otolaryngology to reduce insertion forces during soft-surgery cochlear implantation [6]. Moreover, formulations of HA and its derivatives are discussed in recent literature as transdermal [7] and injectable [8] vehicles for the controlled and localized drug delivery. In this regard, we consider HA as a promising candidate for the provision of implants with a highly biocompatible lubricious surface.

To build up polymeric coatings to surfaces in a controlled manner, an approach was developed about 20 years ago based on alternated deposition of polyanion and polycation layers leading to polyelectrolyte multilayer films (PEM) [9]. For conventional PEM systems, the driving force of the build-up is the alternate overcompensation of the surface charges appearing after each new oppositely charged polyelectrolyte deposition. The thickness of a PEM depends on the pH value and the ionic strength of the solution used during film buildup.

As a first step in the development process, we investigated the coating parameters for the build-up of a lubricious coating based on HA via layer-by-layer deposition. We varied the coating process from Vodouhe et.al. [9] by adding the homopolymer poly(L-lysine) (PLL) or the corresponding monomer, the essential amino acid L-lysine (LL), as electrolytic opponents to HA. Furthermore, we coated the model polymeric implant surfaces with pure HA.

In this study, we present the coating morphology, as well as the coating thickness, as first parameters in order to evaluate the practicability of the different coating compositions.
2 Experimentals

2.1 Materials

All chemicals were purchased from Sigma-Aldrich (Taufkirchen, Germany), Mallinckrodt Baker (Griesheim, Germany), SERVA Feinbiochemica (Heidelberg, Germany), Thermo Scientific (Karlruhe, Germany), or Merck (Darmstadt, Germany) in p.a. quality or higher if not indicated differently.

Hyaluronic acid (HA) (hyaluronic acid sodium salt from Streptococcus equi, Mw=1,500,000 g/mol, <1% protein impurities, CAS: 9067-32-7), poly(L-lysine) hydrochloride (PLL, CAS: 26124-78-7) and L-lysine (LL, CAS: 56-87-1) were also purchased from Sigma Aldrich (Taufkirchen, Germany).

2.2 Build-up of HA, HA/LL and HA/PLL multilayer films via layer-by-layer deposition

With the purpose to enhance the coating adhesion and on the other hand the wettability of the polymer surface samples were treated by O2-plasma in order to generate free hydroxyl groups at the surface. 45 W and 3 min treatments were applied with a radio frequency plasma generator (frequency 13.56MHz, Diener electronic GmbH & Co. KG, Ebhausen, Germany) at a low pressure of 0.3 mbar. For the pure HA coating an NH3-plasma activation was used at 40 W for 2 min at the same frequency to achieve an improved adhesion of the coating.

 Afterwards, the PEM were deposited according to Vodouh et.al. [8] using an automated multivessel dipping robot (KSV Nima Dip Coater multivessel medium, LOT-QuantumDesign, Darmstadt, Germany) to polymer samples. Briefly, polyelectrolyte solutions (1mg/mL) were prepared by dissolving adequate amounts of polyelectrolytes in 0.15 M NaCl solution at pH=6.5. The pH value was adjusted using either 0.1M NaOH or 0.1M HCl solution. At pH 6.5 PLL or LL has a positive charge (cation) while HA is negative (anion).

For the PEM build-up, the samples were firstly dipped into the polycation (LL, PLL) solution for 10 min. Secondly, a rinsing step was performed by dipping the substrates for another 10 min in a 0.15 M NaCl solution at pH=6.5. The polyanion (HA) was deposited in a similar way. The build-up process was repeated until a deposition of n=30 PEM was achieved. For the pure HA coating, the samples were only dipped into the HA solution 15 times and once into a rinsing solution. The residence time was set for 10 min each. After the last rinsing step, all samples were dried at 22°C ± 2°C overnight.

2.3 Evaluation of coating morphology via scanning electron microscopy

Coating morphologies were examined in a Philips XL 30 Environmental Scanning Electron Microscope (Philips ElectronOptics, Eindhoven, Netherlands) operating in the ESEM mode with a water vapor pressure of 1.2 mbar. The accelerating voltage was fixed to 10 kV and the beam current to 11 µA. Working distance was adapted to each sample and varied from 11.4 mm to 18.2 mm, indicated as WD in the legend of each micrograph (Figure 2). Samples were attached to the specimen mount, as obtained after the coating process, and examined at four different positions on the coating.

2.4 Evaluation of coating thickness via confocal microscopy

The evaluation of the coating thickness was performed with polymer samples, which have been sputtered with gold (Agar Sputter Coater, Agar Scientific, Essex, UK) 0.2 bar, 15mA, 120 s, argon atmosphere) prior to the whole coating procedure described previously. This allows for better differentiation between the polymer surface and the hydrogel. Since morphological comparison to hydrogel coatings, applied to bare polymer samples, does not reveal any differences, this modification was assumed to be appropriate.

The coating thickness was measured with the microscope LEXT OLS 3000 (Olympus, Hamburg, Germany). We determined the coating thickness at two positions by ten different measurements applying the objective Plan Achromat MP lan Apo 100x numerical aperture 0.95. A refractive index of 1.5 was assumed for the different coatings.

3 Results and Discussion

The dipping process was feasible for all three modifications. With every coating substance, we could achieve a more or less thick and homogeneous coating.

The representative micrographs from the morphological analysis, Figure 1, show that we generated homogeneous surfaces without any blemishes, cracks or other scratches, which are hardly differable from the polymer surface. Therefore, we scratched the coating surface specifically to increase the visualization of the different coatings. Especially, the PLL/HA coating was clearly visible after the scratching. The LL/HA as well as the pure HA coating appeared slightly thinner than the PLL/HA coating.

We assume that the homogenous morphology is a result of the coating process. Due to the recurrent dipping into the aqueous solutions the coating build-up after one dip is very small. In fact, the high amount of very thin layers form a homogenous coating appearance. The following measurements of the coating thicknesses corroborate these observations. Figure 2 shows representative height profiles of the different coatings. We measured a homogenous coating thickness of 0.6 µm to 0.7 µm for the pure HA coating (position 1: 0.77 µm ± 0.04 µm, position 2: 0.74 µm ± 0.07 µm), 0.7 µm to 0.8 µm for the HA/LL coating (position 1: 0.78 µm ± 0.04 µm, position 2: 0.77 µm ± 0.11 µm) and 2 µm to 2.5 µm for the PLL/HA coating (position 1: 2.09 µm ± 0.39 µm, position 2: 2.33 µm ± 0.19 µm).
We assume that the larger PLL molecule is responsible for the higher coating build-up during the dipping procedure. Furthermore, it appears that a build-up plateau with the LL/HA and pure HA coating was reached, because a double of LL/HA-PEM or HA-monolayers (n=60) did not result in higher coating thickness (data not shown).

As mentioned, the pH value, as well as the concentration of the coating solution, can influence the coating build up. Therefore, we presume that a higher concentration of HA in the solution and pH values of about 10 for the LL or PLL solution and pH=2 for the HA solution might enhance coating build-up during the process [10]. Future investigations have to show a correlation between the coating thickness and the frictional coefficient, so that the coating process can be further optimized.

In addition, we consider crosslinking the HA, as shown in former studies [11]. At this point, additional investigations have to show how the crosslinking would influence the water solubility of HA. This may be important regarding a possible damage of the coating during the insertion into the patient, which in turn will cause a release of particles. Non-crosslinked particles will dilute in the blood immediately, whereas crosslinked particles might not. Furthermore, crosslinked particles can provoke complications, depending on the size of the particles lost.

4 Conclusion

In this study we present three different solutions for coating an implant model surface with a highly biocompatible lubricious coating based on HA and LL or PLL, respectively. Both, coating morphology and coating thickness are very homogeneous for all three presented coatings. Further studies have to show a correlation between the coating appearance and the coefficient of friction.

5 References

Method for Testing of Hydrogel Sensor Coatings

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Keywords: Hydrogel, Biosensor Coating, Test Method, Zirconia.

Abstract
For the integration of sensor components into ceramic-based implants it is of importance to create a reliable sensor-tissue interface. But an implantable sensor interface requires a set of properties: Such as biocompatibility, antifouling properties bio-stability and ion permeability. Hydrogels in general have promising qualities towards an application in this context. To screen different hydrogel coatings a method is needed which allows testing a broad range of materials in relation to the implants' other components. This is not always possible at an early stage of research. Therefore a test chip was developed enabling a parallel screening of hydrogel sensor coatings towards bio interaction and the testing of technically relevant properties using implant material.

1 Introduction
For the integration of electrochemical sensors into ceramic-based implants it is desirable to have the sensor interface closely connected to the surrounding tissue. Due to fibrosis and other biofouling mechanisms this connection is rapidly blocked after implantation. Protective coatings to prevent fibrosis always have an impact on the signal quality. It is a subject of current research to minimize this influence [1, 2]. A sufficient lifetime is important but biocompatibility is the key-property of this kind of sensor coating. Specially designed hydrogels combine these key-properties and are for that reason well suited for an application as sensor coatings [3]. This makes iterative test cycles necessary which tend to be time consuming and costly. If test chips made from a third material (such as glass) are used for an early coating screening the transfer to the actual material might lead to problems with adhesion and coating processes. With a test chip based on medical grade implant ceramics innovative sensor coatings can be tested very close to the intended application and in interaction with the technical interfaces at an early stage of research. Figure 1 shows the biotestchip that was used for testing of different kinds of hydrogel coatings. The chip fits into a standard twenty-four-well cell culture dish which makes it suitable for parallelized biocompatibility testing. Further it has a screen-printed interdigital silver structure on the front side with in-plane connection to contact pads on the backside and can be used for a set of different experiments: The silver can be used as an indicator of the interaction between a biological surrounding and the technical interface through a coating. Further the chip can be brought into a special test chamber where the coated front side is exposed to different fluids whereas the electrical connections of the backside are read out to evaluate the sensor functionality.

2 Methods
Biotestchips made from medical grade zirconia 15 mm in diameter and with pre-drilled vias were obtained from Moje-Implantate GmbH & Co. KG, Hermsdorf, Germany, in larger quantities. Via-filler and screen-print silver paste were used from DuPont™ Microcircuit Materials. The vias were filled serially using a micro-placer equipped with a pneumatic syringe whereas the screen-printing was carried out using a laser-cut template permitting the screen-printing as a parallel batch process.
All used hydrogel coatings were supplied by Innovent e.V., Jena, Germany. Different coating methods were applied such as spin- and dip-coating and molding. Figure 2 shows schematically the test regime for the screening of sensor coatings: For the bio testing of the respective coatings the coated test chips were autoclaved, brought into twenty-four-well cell culture dishes and cultivated with L-929 mice fibroblasts for seven days at the Institute for Bioprocessing and Analytical Measurement Techniques (iba), Heilbad Heiligenstadt, Germany. Tests were repeated at the Center for Micro- and Nanotechnologies (ZMN) at the Technische Universität Ilmenau, Ilmenau, Germany. The influence of autoclaving and cell culture medium on chips and coatings were tested separately.

3 Results

The test chips were used for the testing of protective coatings ideally expressing high ion permeability. The influence of the sterilization and the cell testing upon the silver sensor structures underneath the hydrogel has been examined microscopically.

![Figure 3: Microscopic image of a coated and an uncoated silver structure after bio testing. Right: The uncoated sample is oxidized by the cell culture medium. Left: A transparent antifouling coating prevented the silver structure from being oxidized. Scale bar: 200 μm.](image)

Fig. 3 shows an example of a protected (by a hydrogel coating) and an example of an unprotected silver structure on a test chips’ surface after bio testing. It is visible how the silver electrodes are oxidized by the compounds of the cell culture medium without being protected by the hydrogel. The silver electrodes have an influence on the viability and metabolism of the cell culture, which eventually can be depleted by a protective coating. The impact of the coating on the signal, mostly a decrease in amplitude, was measured within the test chamber. The lower this influence, the better a hydrogel is suited for a passive sensor coating. Figure 4 shows the measured conductivity through different coatings after the chips have been exposed to a cell culture experiment for seven days. In the chart: Blank represents the uncoated sample, A and B are examples of different hydrogels which were spun onto the test chips at different thicknesses (A, A_2). B shows better signal transmission properties than A also for thicker layers whereas the transmission through A strongly depends on the layer thickness. For a better understanding the values were normalized to the ones of the uncoated samples.

![Figure 4: Relative Conductivity measured through biotestchips with different coatings (A and B) at different thicknesses (A, A_2) after biotesting for a test chamber filled with 0.1 mol/l potassium chloride solution. The values were normalized to the uncoated sample (Blank).](image)
4 Conclusion

Screen-printed conductor structures and electrical feed-throughs could be integrated into implant ceramics to create test chips for sensor coatings. These allow, using the same test chips, determining the sterilizability, biocompatibility and adhesion of protective and transducer sensor coatings in relation to the actual materials and components of a future implant. Furthermore it is possible to use pre-aged coatings for experiments on the transducer properties and layer specific features. In cell culture the test chips can be used in standard dishes for parallelized screenings. The test method is suitable to predict and prevent problems that may occur during the assembling and testing of a final device in an early stage of development.

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6 References


Complex bacteria-cell-implant co-culture model for implant screening

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Introduction

The application of dental implants in partially or fully edentulous patients revolutionized dentistry. However, implants are not resistant to failure. Chronic infection of peri-implantary tissue is one possible reason, since dental implants as natural teeth are prone to bacterial biofilm formation. Fundamental differences constitute implants more susceptible than natural teeth (Belibasakis GN 2014). Therefore many research groups are developing innovative biomaterials. So far new implant materials are usually examined regarding the interaction with oral bacteria (antimicrobial activity) or human cells (cytocompatibility) (Pfaffenroth C et al 2011), whereas other groups are more interested in the interaction between cells and bacteria (Guggenheim B et al 2009). For the first time we would like to combine in vitro all three aspects in one complex screening model testing dental implants simultaneously for their antimicrobial activity and cytocompatibility prior to pre-clinical animal studies.

Methods

For a complex 3D-model different components of the system have to be established separately before combined. As a stabilizing matrix different hydrogels were tested regarding support of different peri-implant tissue cell types. In parallel cell-biofilm co-cultures were tested in order to gain more knowledge about conditions, which enable a middle- to long-term co-culture. Cell adhesion quality on implants was determined by focal adhesion point quantification. Finally, the effect of biofilm on the cell-implant bond will be visualized in the complex 3D-model using Scanning Laser Optical Tomography exploring the whole implant surface over time.

Results

Preliminary tests showed that particular commercial available hydrogels were able to support human gingival fibroblast adhesion and growth. Furthermore, artificial biofilm culture on filter membranes was successful with high bacterial viability.

Conclusion

Complex screening of new implant materials turns out to be important since an antimicrobial effect is desired in combination with the essential biocompatibility. Moreover, a complex bacteria-cell-implant interaction in vitro could generate more detailed and relevant information to improve implant design, minimizing peri-implant infections before proceeding to in vivo experiments.
Influence of different nano-structured surface topographies on electrode impedance

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Introduction

A broad range of biomaterials is used in today’s medicine. These biomaterials such as electrodes for high frequency deep brain stimulation interact with the neural tissue. An important role in this interaction concerns the surface topography of materials. For example a nano-structured surface may affect impedance thus leading to improved electrophysical behaviour.

Methods

For experimental applications bipolar stimulation electrodes were prepared manually by isolated pairs of Pt-Ir wires (75 µm) glued at both ends of a guide cannula (24G). At one end electrode tips were formed by removing isolation and adjusted to 500µm of length. At the opposite end gold-plated contact sleeves were soldered. Impedance of electrodes was tested both at a fixed frequency (200 Hz) and dynamically by means of impedance spectroscopy (64 Hz-50 kHz) before and after nanoparticle coating. Nano-coating was achieved by electrophoretic deposition of Pt-nanoparticles set to 5, 7 and 50 nm diameter after conditioning the electrode tips in nitric acid. In one electrode batch a mixture of 7&50 nm particles was examined.

Results

Differences in impedance were found especially with respect to particle size: coating with small nanoparticles (5 and 7 nm) increased the average impedance (109%, 46%, respectively) whereas large particles (50 nm) showed virtually no change (0.3%) in impedance. Coating with a composite of nanoparticles (7&50 nm) was decreasing impedance (-29%) in comparison to the uncoated state.

Conclusion

So far, electrode impedance seems to be inversely proportional to the particle size. This simplification, however, does not explain the drop in impedance when coating with a nanoparticle compound of different size. Also not yet resolved is the coating’s influence on changes in the resistive and capacitive configuration of electrode impedances.
**Nitinol thin film for endovascular application**

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**Introduction**

Application of thin film technology in the field of endovascular devices, such as filters, stents and grafts, has been hampered i.a. by limitation of film thickness and lack of data in mechanical and fatigue performance. Recent data suggest that Nitinol thin film structures can reliably be produced up to approximately 70µm thickness, this allowing new complex geometries and micro structuring for enhanced biocompatibility. The author presents the feasibility and performance of thin film stent structures as a novel technology for advanced miniaturized endovascular devices.

**Methods**

Based on design of stents for neurovascular application, initial lab prototypes have been manufactured for mechanical comparison with conventional manufactured stents. Furthermore, flow and vessel optimized strut geometries, which are made possible by use of thin film technology, were realized. Mechanic, fatigue behaviour and surface properties were tested in vitro. A first in vivo evaluation of thrombogenicity and endothelialisation was performed.

**Results**

Superior structure deformation and fatigue behaviour have been showed compared to conventional Nitinol devices, this suggesting an enhanced delivery through microcatheters, expansion and vessel apposition under pulsatility. First results in regard of in vivo vessel patency and endothelialisation will be presented.

**Conclusion**

The possibility to obtain complex geometries and micro structuring using thin film technology opens a wide range of application in the endovascular field. Moreover, it offers a significant future potential for manufacturing devices in further biomedical areas like neurostimulation, electrophysiology and cardiac rhythm management.
Differently Functionalized Silica Nanoparticles – Cytocompatibility and Antimicrobial Effect

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Introduction

Spherical silica particles produced by flame pyrolysis are commonly used filler particles for dental composites but show poor drug loading capacities. In the present study, nanoporous silica nanoparticles (NPSNPs) synthesized via sol-gel chemistry, which exhibit higher surface area, larger pore volume and hence better drug delivery properties, were used. Antimicrobial agents incorporated into the nanopores of these particles are gradually emitted over time. In this study, NPSNPs were differently functionalized either to increase the loading capacity or to prevent agglomeration. Chlorhexidine digluconate was chosen to load into the particles as it is successfully employed in dentistry. The aim of this study was on the one hand to assess the cytocompatibility of these silica nanoparticles and on the other hand to prove their antibacterial properties.

Methods

The cytocompatibility was examined by analyzing the morphology and metabolic activity of gingiva fibroblasts. Therefore, SEM analyses and MTT assays were applied after incubation with different concentrations of NPSNPs. To check for antibacterial properties, the nanoparticles were incorporated into agar plates and colony formation of clinical relevant bacteria was assessed. For more detailed investigation the influence of NPSNPs on biofilm formation and mature biofilm was evaluated using resazurin-based viability assay and confocal laser scanning microscopy.

Results

Up to a concentration of 25 µg/ml NPSNPs gingiva fibroblasts showed no decrease in viability. Furthermore, the NPSNPs inhibited bacterial growth already at 12.5 µg/ml. This depicts a therapeutic window of 25 – 12.5 µg/ml. These concentrations were also suitable to negatively influence biofilm formation.

Conclusion

It was shown that different NPSNPs are able to inhibit bacterial growth without disturbing cell viability, which makes them a promising tool for antibacterial dental materials. Further investigations toward clinical application need to verify their cytocompatibility, address their antibacterial effect when incorporated into composites and possibilities to make their emission stimulus-responsive.
Modified Chandler Loop system for dynamic hemocompatibility testing of vascular implants

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Abstract

The development of hemocompatible biomaterials and vascular implants is one of the biggest tasks in biomedical engineering. Due to the fact that there are no standardized test setups a modified CHANDLER LOOP system was designed for the in vitro testing of biomaterials and cardiovascular implants. The modified system consists of disks, which serve as carriers for tubes or vascular implants. They are placed in a rig and are secured by locking pins, which are attached to the locking device. The rig is mounted in a water bath and rotated by an electric motor. By this technique up to 20 samples can be tested simultaneously. The modular design leads to an improvement in handling compared to other similar systems. The disk concept ensures perfect circularity of the tubes and undisturbed flow conditions during testing. The system includes built-in sensors capable of controlling and recording rotating velocity and temperature throughout test series. A study was designed to validate the new in vitro system by comparing the results to an established system at the institute¹. Therefore medical tubes are partly filled with anticoagulated blood and rotated for 60 minutes in each system. The study focused on an improvement of handling and background activation. The mounting duration decreased up to 56% compared to the established system. The decrease of thrombocytes was similar with about 12% in both systems. The highest rate of hemolysis was 0,21% for the modified and 0,1% for the established system. The fast removal of disks enables time dependent test series with short standing times. The low background activation and improved handling in combination with the measurement technique increases the reproducibility of test series and qualifies the modified CHANDLER LOOP System for the testing of cardiovascular implants and materials.

1 Introduction

Cardiovascular diseases are responsible for 50% of the causes of death in Europe². This high morbidity rate demonstrates the importance and the demand for cardiovascular implants. Their development is one of the biggest challenges in biomedical engineering. The limited hemocompatibility of materials used for cardiovascular implants and other blood carrying devices shows the lack of perfect synthetic products. Poor hemocompatibility can cause thrombosis, a destruction of red blood cells called hemolysis or an activation of the coagulation system. For research and development purposes of cardiovascular implants and devices, dynamic in vitro test stands provide a cost-effective method to test new prototypes and materials under physiological conditions (Figure 1). DIN EN ISO 10993-4 states different parameters that should be investigated when examining new products, but does not state construction specifications of test stands³⁴. This leads to a big variety of test stands and to a lack of comparability between different research groups. A modified CHANDLER LOOP System was designed to increase the reproducibility of test series and was equipped with measurement technology to monitor rotating velocity and temperature. The handling of the systems was simplified to reduce user dependent errors, which could have a negative influence on the test results.

1.1 In vitro testing of hemocompatibility

Regarding to the licensing procedure of medical devices, such as stents, tubes or cardiovascular prosthesis, in vitro tests offer good opportunities to analyze new products in respect to bio- and hemocompatibility. In DIN EN ISO 10993-4 guidelines are illustrated concerning the hemocompatibility testing of blood-contacting biomaterials. Parameters of the test setup like temperature, flow and other physiological conditions should be as close as possible to the conditions in the future field of application.³⁴ CHANDLER developed a system, which inspired the development of hemocompatibility test setups. By rotating tubes partly filled with whole blood at an angle of 23° on a disk CHANDLER was able to analyze the function of the coagulation system⁵⁶⁷. Nowadays many hemocompatibility test setups are based on CHANDLERS set up. Modified CHANDLER LOOP setups maintain the samples at 37°C to imitate physiological conditions.

Figure 1 Established CHANDLER LOOP system at the Institute for Multiphase Processes¹.
1.2 Modified Chander-Loop-System

The modified system consists of disks fabricated from PMMA, a rig, a locking device and sensors to measure rotating velocity and temperature (Figure 2). For testing purposes tubes are placed in the disks, which ensure a perfect circularity of the tubes during testing and handling.

Figure 2 Left: modified CHANDLER LOOP construction (shown without water bath and motor). Right: Standard disk.

Up to 20 of these disks can be placed in the rig, which is placed in a water bath and driven by an electric motor. The water bath is heated by a compact laboratory immersion heater so that no bulky heating circulator is needed (Figure 3). Disks are easily secured by a locking device, which can be operated within seconds. Two ball bearings outside the water bath and one slide bearing inside the water bath are used to mount the construction. Temperature and rotating velocity are permanently measured and displayed on an LCD screen and saved to an excel file for documentation purposes as well. The hardware used to measure rotating velocity and temperature consists of an Arduino Uno, a hall sensor (TLE 4945 L) and a temperature sensor (DS18B20). The program to read and display the data was written in C/C++.

Figure 3 Modified CHANDLER LOOP shown with water bath, immersion heater and blood filled tubes.

The modular construction of the CHANDLER LOOP provides a significant improvement of handling. Single samples can be removed in less than 10 seconds in a random order, which is important for time dependent test series. Loading and unloading from the top avoids water contact for the user and improves the handling even further. The standard disk allows a testing of tubes with an inner diameter of 2.4 mm or 4 mm and a length of 680 mm or 580 mm. Besides tubes the modified CHANDLER LOOP enables the testing of cardiovascular stents, cardiovascular prosthesis or artificial heart valves. Furthermore the compactness of the new system enables easy transport and leads to a high degree of mobility.

2. Materials and Methods

A study has been designed to compare the new modified CHANDLER LOOP system (A) with a validated system used at the institute (B). Besides comfort in handling the biggest difference between the systems concerns the perfect circularity of the tubes during testing as well as the installed measurement technique. The established system uses six stainless steel pins to hold the tubes (Figure 1). This can cause diminution of the diameter, which can be followed by a change in flow conditions and dead water zones.

Citrated porcine blood from six different individuals was used for this study. Three individuals were tested on each of the two test days. To compare the systems each sample was run in system A and system B. Tubes used in this series had a length of 680 mm and an inner diameter of 2.4 mm (Saint Gobain Performance Plastic, Tygon® S-50-HI), they were filled to a level of 440 mm which is equal to a blood volume of 2 ml. The ends of the loops were closed end to end with silicone tubes to minimize blood irritation and avoid dead water zones. Both systems were set to 37°C and 18 rpm which is equal to a velocity of 0.2 m/s. After the test duration of 60 minutes, blood parameter such as hematocrit, hemolysis, thrombocyte count and coagulation time were analyzed. To determine the hematocrit, whole blood was filled into capillaries and was centrifuged (parameters: 11,000 x g, 4 min). The comparison to the zero values allows a nullification of physical variations and improves comparability between the two test days. DIN EN ISO 10993-4 states the measurement of hemolysis, as a standard parameter of dynamic hemocompatibility tests. To evaluate the index of hemolysis the following equation was used:

\[ IH = \left( 1 - \frac{HCT}{100} \right) \times PHb \times 100\% \]

- IH = Index of hemolysis [%]
- HCT = Hematocrit [-]
- PHb = Plasma hemoglobin [g/dl]
- THb = Total hemoglobin [g/dl]

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Then the coagulation time was measured using a coagulation time analyzer. The total hemoglobin (THb) indicates the amount of hemoglobin in the whole blood. The amount of hemoglobin in the plasma is named plasma hemoglobin (PHb). If membranes of red blood cells are damaged hemoglobin is released, which is reported by an increase of plasma hemoglobin. Both parameters were measured using a Photometer.

To determine the activation of the coagulation system the number of thrombocytes of each sample was counted and compared to the zero values using the following equation.

\[ TI = \frac{TC_x}{TC_0} \times 100\% \]

- \( TI \) = Index of thrombocytes [%]
- \( TC_x \) = Thrombocyte count at point x [1/µl]
- \( TC_0 \) = Zero value [1/µl]

3. Results

No remarkable increase regarding the index of hemolysis neither in the modified nor in the established CHANDLER LOOP system could be observed. The highest increase of hemolysis was 0,21% for the modified and 0,1% for the established system. There was a slight difference of the coagulation time between the two systems, it decreased by 12 seconds in the modified system and by 9 seconds in the established system, in average, compared to the zero values with an average of 91 seconds (Figure 4).

The decrease of thrombocytes within one hour is shown in Figure 5. Comparing the modified and the established system there is no remarkable difference in the thrombocyte count. A decrease of 12,87% could be observed for the established and 11,91% for the modified system.

![Figure 5 Index of thrombocytes after 60 minutes, 87,13% in the established system and 88,09% in the modified system; (n=6).](image)

A final analysis regarding the coefficient of determination in the thrombocyte count shows a match of 90,5% between the modified and the established system (Figure 6).

![Figure 6 Analysis regarding the coefficient of determination between the two systems in perspective to the thrombocyte count after 60 minutes.](image)

The handling with the modified system was sensed as more comfortable and easier compared to the established system. Mounting time with the modified system decreased 56% compared to the established system.

4. Conclusion

The modified CHANDLER-LOOP system was designed to perform as an compact and easy to operate in vitro hemocompatibility test stand. It is equipped with measurement technique to control and document test parameters. To validate the modified system a test series was performed to compare the modified and the system used previously with regard to the background activation and handling comfort.

The minor changes regarding the rate of hemolysis and the low decrease in thrombocyte count of both systems proof a low background activation of the coagulation system and a very small destruction rate of red blood cells. This leads to
a high sensitivity of both systems for the testing of vascular implants. The similar test results of the two systems regarding the parameters thrombocyte count, hemolysis, coagulation time, hematocrit and the high coefficient of determination regarding the thrombocyte count show that the modified system can also be used as a test setup for dynamic hemocompatibility tests.

The improved handling of the modified system enables time dependent test series with short interruptions for sample takings. The integrated rotating velocity and temperature control enables the supervision of both parameters during the test duration and the logging function makes it possible to record both parameters for documentation purposes. The quicker handling as well as the rotating velocity and temperature control leads to a reduction of the error rate and an improvement in reproducibility of test series. The modular disk concept enables the testing of a big variety of implants such as cardiovascular prostheses, stents or heart valves.

5 References


Ultrahigh molecular weight polyethylene reinforced with quasicrystalline particles for biomedical applications

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Abstract

In this study, the influence of quasicrystalline particles on the properties of the ultrahigh molecular weight polyethylene-based composites was investigated. The composites were prepared using a combination of mechanical alloying (MA) and hot processing by spark plasma sintering (SPS). Wide angle X-ray diffraction (WAXRD) was employed to investigate the uniformity of the sintered parts at a structural level. Nanoindentation tests were further performed to determine hardness and elastic modulus.

1 Introduction

Ultra-high molecular weight polyethylene (UHMWPE) is used in orthopaedic implants due to its favourable physical properties: easy thermoforming, great machinability and resistance to wear and ageing. In spite of the low wear behavior of UHMWPE compared to other polymers, wear remains a major problem in total joint replacements because it means loosening of implant components [1]. This further limits the life expectancy of hip prostheses. It was reported that quasicrystalline fillers may significantly improve mechanical properties in polymer-based composites [2–4].

A large number of papers have dealt with the preparation of different composite materials through mechanical alloying [5–11]. However, there are only a few papers concerning the production of polymer composites containing quasicrystalline fillers with the use of the mechanical activation process. The essence of the method consists in mixing a soft polymer matrix with hard particles of a reinforcing phase on the microscopic structural level in order to ensure a uniform distribution of particles. In this part of the study, UHMWPE was chosen as a basic material for the preparation of polymer nanocomposites reinforced with quasicrystalline particles.

2 Methods

2.1 Sample preparation

Quasicrystalline-polymer composites were generated starting from UHMWPE and Al-Cu-Fe quasicrystalline alloys in powder form. UHMWPE was purchased from DSM Biomedical (Netherlands) and was used as received. Quasicrystalline alloys were prepared by mechanical alloying and subsequent hot processing.

2.1.1 Mechanical alloying

Mixtures of UHMWPE and Al₁₆Cu₂₃Fe₁₀ quasicrystalline powders were weighted, added together and mechanically alloyed using a RETSCH PM 400 planetary ball mill. The quasicrystalline weight fraction was varied between 10 and 40 wt%. The powders were mechanically milled for 2 hours.

2.1.2 Spark plasma sintering (SPS)

The composite powders were further SPS processed in vacuum, under a uniaxial pressure of 7 MPa. A pulsed DC voltage was used. Any degradation of the surface modification may have serious impact on the composite properties, so the temperature was at first raised to 170°C at a fast heating rate of 40°C/min followed by a short dwell-time of 60 s at this sintering temperature in order to avoid any degradation.

2.2 Characterization methods

2.2.1 Scanning electron microscopy (SEM/EDX)

The SEM images were acquired in low vacuum mode, using a Philips XL 30-FEI microscope operating at 10 kV, spot size of a few nanometers and a working distance of 10 mm.

2.2.2 Wide angle X-ray diffraction analysis (WAXRD)

The WAXRD measurements were performed with a D8 Bruker AXS with a General Area Diffraction System (GADDS). This setup delivers a well monochromatized and parallelized X-ray beam of the CuKα wavelength λ_{Kα} = 1.54178 Å. The working conditions for the diffractometer were 40 kV and 40 mA.

2.2.3 Nanoindentation

Hardness and elastic modulus were calculated from the load-displacement data obtained by nanoindentation using a Nano Indenter UNMT (Universal Nano/Micro Tribo-meter, CETR) with a Berkovich indenter.
3 Results

Image 1 shows a SEM image depicting the morphology of the SPS sintered quasicrystalline-polymer composite. SEM micrographs of the sintered pellets indicate a complex microstructure, with no porosity, where different extents of both intercalation and exfoliation are generally observed. In some areas, the forces of interaction between the quasicrystalline particles could not be totally dissolved and there are some small regions where the particles are closer together, but no agglomeration was observed.

![SEM micrograph at the surface of the ACF/UHMWPE sintered pellets.](image1)

Image 1 SEM micrograph at the surface of the ACF/UHMWPE sintered pellets.

WAXRD was employed to investigate the uniformity of the sintered parts. The diffraction patterns were collected from the inner surfaces of the pellets. The WAXRD patterns (Image 2) reveal the presence of nine peaks distinctly associated with the pure quasicrystalline phase (φ-icosahedral) and three peaks given by the orthorhombic UHMWPE.

![WAXRD pattern for the Al₆₇Cu₂₃Fe₁₀/UHMWPE composite after SPS at 170°C with an applied force of 7 MPa.](image2)

Image 2 WAXRD pattern for the Al₆₇Cu₂₃Fe₁₀/UHMWPE composite after SPS at 170°C with an applied force of 7 M Pa.

The nanoindentation measurements are shown in image 3. For each material, two or three load-displacement curves were measured. The results revealed that the Al-Cu-Fe/UHMWPE composites show better mechanical properties than neat UHMWPE. Both hardness and Young’s modulus of the Al-Cu-Fe/UHMWPE composites (H= 66 ± 15 MPa, E = 2 ± 0.7 GPa) were found to be higher compared to the UHMWPE (H= 44 ± 4 MPa, E = 1.3 ± 0.1 GPa).

![Hardness and Young’s modulus.](image3)

Image 3 Hardness and Young’s modulus.

4 Conclusion

Composites of UHMWPE and different weight percentages of Al-Cu-Fe quasicrystalline particles were prepared by wet mixing. SPS results in homogeneous pellets with uniform ultrafine structure without grain growth. The phase composition analysis reveals the presence of pure icosahedral phase and orthorhombic polyethylene, which leads to the conclusion that the high heating rates applied in the sintering process have no influence on the phase transformation sequence. Nanoindentation tests revealed that the composites show a remarkable increase of the hardness and Young’s modulus compared to the initial polymer.

References

Study of Magnesium Degradation in Porcine Blood Plasma

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Keywords: biomaterial/ magnesium alloys/ magnesium degradation / photometrical measurement

Abstract

For applications of degradable magnesium implants in medicine the knowledge of the degradation is very necessary. There are many methods to investigate the degradation process. In this study a method especially for small magnesium samples and low degradation rates are evaluated. Therefore seven magnesium alloys were immersed in plasma for 45 days. The magnesium emitted by the sample during degradation process was measured photometrically. These values were compared with the weighted mass loss which is a commonly used method to determine the degradation. The results show that the photometrical measurement of the degradation is an adequate method and allows a time-dependent investigation of degradation.

1 Introduction

Magnesium and its alloys are promising materials for medical application due to three special properties: Firstly, the mechanical properties of magnesium are closer to the mechanical properties of bones than every other metallic material. Especially Young’s Modulus is similar to that of bones. Consequently orthopaedic implants of magnesium can avoid stress shielding which is an often appearing complication. Stress shielding is the result of the absence of mechanical stimulation due to a very stiff implant material. It leads to degeneration of bone tissue around the implant. Secondly, magnesium and magnesium alloys degrade in the body because of their low chemical resistance to fluids containing water and ions. Controlled degradation can be useful in medical care of bone injury because screws or platters do not need to be removed after the healing process is finished [1]. Thirdly, magnesium is an essential substance in body metabolism and therefore intoxic. It rather seems to support the bone healing process [1].

Previous studies have shown that the challenge consists in predicting the behavior of magnesium material, like the degradation rate and the degradation products, in the body. Witte et al. [2] demonstrated that the degradation rates of magnesium alloys under ASTM (American Society for Testing and Materials) conditions, in NaCl-solution, cannot be reproduced by in vivo experiments. The alloys in their in vivo experiment had a much lower degradation rate and showed very different behavior than the alloys under in vitro conditions. Following this and other results, it is clear that the influence of the medium is considerably strong. Hence, many researchers use in their experimental studies SBF (Simulated Body Fluids) which have a similar ion composition and concentration to body fluids. Schenk [3] showed that the degradation products are not only magnesiumhydroxid and hydrogen, but also Apatite and Carbonates. A stable film forms on the metallic surface which has a protective effect to the metal. Liu et al. [6] discribed in their study that the inhibiting effect of the SBF increases by adding albumin. Albumin is one of the principal proteins found in plasma. Fig.1 shows the difference between degradation in SBF and blood plasma.

For representative degradation studies it is of utmost importance to simulate the body conditions as realistic as possible [1]. The degradation is quantified and qualified with many different methods. The electrochemical measurement gives a good indication to the mechanism of magnesium degradation but only permits limited conclusions about the degradation rate [4]. One of the degradation products is hydrogen which enables the calculation of the degradation rate from the emission of hydrogen. But the high diffusibility of hydrogen leads to a large measurement error especially for little samples and small degradation rates [5].

In the present study the physiological environment is simulated with blood plasma which is a body fluid containing proteins, ions and a pH-buffer system. So it is relatively close to physiological conditions. Because of the difficulties to measure the degradation parameters with hydrogen an alternative method based on photometrical measurements of magnesium ions in plasma is evaluated in this study. This method enables the observation of the degradation during the complete period of investigation.

Fig. 1: Magnesium samples after degradation in r-SBF (l.) andin blood plasma (r.).
2 Materials and Methods

Seven different magnesium alloys were investigated. Table I gives the alloys as well as their masses at the beginning of the experiment. Five samples of every alloy were tested to ensure a sufficient statistics. The samples were cylindically shaped (height: 2 mm, diameter: 5 mm). At the beginning of the experiments the samples were weighed (see Table 1). As medium for the degradation test, plasma from a pig was used. Therefore the blood of several pigs was mixed and centrifuged. The plasma was stored at a temperature of approximately -80°C. After defrosting in a water bath, 1 vol.% penicillin was added to prevent bacterial contamination during the incubation. The samples were immersed in plasma-filled 12-well-plates. Each sample was put in 5 ml plasma and incubated in a 5%CO₂-atmosphere at a temperature of 37°C. The pH-value was controlled every day with a pH-meter to prevent an increasing of the pH-value over 8.5.

After three or four days, the samples were washed up with ethanol and distilled water and were placed into fresh plasma. The magnesium content of the plasma (hereafter called “magnesium value”) was detected photometrically using Xylidilblue. Xylidilblue reacts with magnesium ions by changing the color from blue to violet. Therefore a calibration curve which was determined before was used. If necessary, the plasma was diluted with distilled water before measuring the magnesium value. Fig 2. shows the experimental plan. The magnesium value describes the concentration of the degraded magnesium in the plasma. It is calculated as the difference between the magnesium ions in the plasma after and before the incubation. The concentration of magnesium in the plasma is expressed in mg/dl. Consequently, the degradation rate is defined as the concentration of the degraded magnesium per day (mg/dl)/d.

The total immersion time was 45 days. Before the samples were weighed again, an EDX (energy dispersive X-ray spectroscopy) analysis was made to determine the composition of the oxide layer and the samples were treated with chromic acid after ASTM-Standard G1-03 to remove the oxide layer.

Table 1: Samples of magnesium alloy used in the experiments.

<table>
<thead>
<tr>
<th>Alloy</th>
<th>mass before immersion (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MgCa0.8</td>
<td>67.98</td>
</tr>
<tr>
<td>Nd2</td>
<td>68.36</td>
</tr>
<tr>
<td>MgNdLaZr220.5</td>
<td>70.12</td>
</tr>
<tr>
<td>LAE442</td>
<td>65.18</td>
</tr>
<tr>
<td>LANd442</td>
<td>63.34</td>
</tr>
<tr>
<td>La2</td>
<td>68.62</td>
</tr>
<tr>
<td>Cer2</td>
<td>68.60</td>
</tr>
</tbody>
</table>

3 Results

3.1 pH-Control

In the process of magnesium degradation hydroxide ions are emitted into the surrounding medium. A pH-value of 8.5 or higher leads to a stabilization of the magnesiumhydroxid layer and thus inhibits the degradation. During the experiments the pH-value of all samples was constant in a range from 7.4 to 7.8. Thus no inhibition was caused by an increasing pH-value. The physiological value of 7.4 (e.g. [7]) is close to the pH-values in the performed experiments.

3.2 Photometric Results

Fig. 2 shows the time-dependent magnesium concentration in the plasma during the complete immersion period of 45 days. A strong increase in magnesium concentration can be seen for the first 15 days. Thereafter, the alloys show a different behavior: LAE442 goes into a quasi stable state where the magnesium degradation is very slow. La2 and
Cer2, however, show a rather linear development and have the highest degradation rates of the examined alloys. The results agree with previous observations made by Menze [10]. He investigated the degradation of pure magnesium in serum during an immersion period of 15 days. In his study the degradation rate of magnesium is 0.83 mg/(dl d). In present study the value of degradation rate which is averaged over all alloys is 1.04 mg/(dl d) for the first 15 days. In comparison with the degradation rate of pure magnesium in SBF (4 mg/(dl d)) the mean degradation rate in serum and plasma is four times slower.

Fig. 3: Magnesium concentration in plasma during the immersion period for all alloys (n=5).

2.3 Mass Loss

In Fig. 4 the weighted mass loss ($m_w$) is compared with the mass loss calculated from the photometrical data ($m_p$). The error bars represent the maximum and minimum values. It can be seen that $m_p$ is always smaller than $m_w$. The differences between all alloys $m_w$ and the corresponding $m_p$ lie in small range between 0.43 mg for LANd442 and 0.96 mg for Nd2. The reason for the systematic difference between the values of $m_w$ and $m_p$ is that the weighted mass loss values contain the mass of the oxide layer. This additionally amount of mass is not considered in the photometrically measured mass loss. However, the photometrical analysis qualitatively agrees with weighted mass loss: Alloys with high magnesium concentration in plasma have a high weighted loss of mass.

For a better comparability, the mean difference $\Delta m$ between $m_p$ and $m_w$ was calculated for all alloys. The mean difference is 0.73 mg. The modified mass loss values of $m_p + \Delta m$ are shown in Fig. 5. The corresponding values of $m_w$ are unchanged. It can be seen that the weighted and the modified photometrically determined mass loss values agree well. The rather small differences of the alloys can be explained by the uncertainties of the measuring systems and by the lack of knowledge of the thickness and composition of the oxide layers. Table 3 lists for better comprehensibility the mass loss data.

<table>
<thead>
<tr>
<th>Alloy</th>
<th>$m_w$ (mg)</th>
<th>$m_p$ (mg)</th>
<th>$\Delta m$ (mg)</th>
<th>$m_p + \Delta m$ (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CaMg</td>
<td>2.72</td>
<td>1.82</td>
<td>0.89</td>
<td>2.55</td>
</tr>
<tr>
<td>Nd2</td>
<td>3.22</td>
<td>2.25</td>
<td>0.96</td>
<td>2.98</td>
</tr>
<tr>
<td>MgNdLaZr</td>
<td>3.12</td>
<td>2.27</td>
<td>0.85</td>
<td>2.99</td>
</tr>
<tr>
<td>Cer2</td>
<td>3.72</td>
<td>3.16</td>
<td>0.60</td>
<td>3.88</td>
</tr>
<tr>
<td>LAE442</td>
<td>1.72</td>
<td>1.03</td>
<td>0.69</td>
<td>1.75</td>
</tr>
<tr>
<td>LANd442</td>
<td>2.84</td>
<td>2.40</td>
<td>0.43</td>
<td>3.13</td>
</tr>
<tr>
<td>La2</td>
<td>4.04</td>
<td>3.39</td>
<td>0.65</td>
<td>4.11</td>
</tr>
</tbody>
</table>

Fig. 4: Photometrically measured and weighted mass loss of all alloys after 45 days of immersion in plasma(n=5).

Fig. 5: Modified photometrically measured and unchanged weighted mass loss (n=5).

2.4 EDX-Analysis

The EDX shows the difficulties to give general quantitative statement about the composition of the oxide layer. As it can be seen in Table 2, the magnesium content in the oxide layer varies considerably between the alloys and is
independent from the mass loss. It seems that every alloy has its individual formation of the oxide layer. Many studies deal with the examination of the oxide layer: Yang and Zhang [8] showed in their study that the oxide layer consists of a kind of apatite. Apatite is a hardly soluble substance containing Ca$^{2+}$, Mg$^{2+}$ and PO$_4^{3-}$. Lui et al. [6], who examined the influence of albumin to the degradation behavior, proved in his study that albumin adheres on the surface and protects the surface from aggressive anions like Cl$^{-}$. This observations are confirm with the results found in this study of a rather high part of carbon (around 25at.%) in the oxide layer.

Table 2: Magnesium contents in oxide film measured with EDX

<table>
<thead>
<tr>
<th>Alloy</th>
<th>Magnesium content (wt%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MgCa$_{0.8}$</td>
<td>6.52</td>
</tr>
<tr>
<td>Nd$_2$</td>
<td>3.33</td>
</tr>
<tr>
<td>MgNdLaZr$_{220.5}$</td>
<td>5.59</td>
</tr>
<tr>
<td>LAE442</td>
<td>7.66</td>
</tr>
<tr>
<td>LANd442</td>
<td>4.71</td>
</tr>
<tr>
<td>La$_2$</td>
<td>3.69</td>
</tr>
<tr>
<td>Cer$_2$</td>
<td>6.27</td>
</tr>
</tbody>
</table>

Fig. 6 shows a further difficulty in quantification the oxide film. The surface of the samples is inhomogeneous. For example the oxide film of the CaMg0.8 sample is cracked and chipped. The composition of these areas is totally different to areas with intact oxide layer. The averaged value of the magnesium content in the oxide layer lies at 4.71at.%. In areas where the layer is chipped, the magnesium content reaches values of 18.71at.%. In connection with the high oxygen value of 46at.% in these areas it stands to reason that there is a layer of magnesiumhydroxid mixed with a small amount of proteins and apatite. Table 4 compares the intact oxide layer with the chipped area shown in Fig.6.

Table 4: Atomic percentage of several element in the intact layer and the chipped layer of CaMg0.8

<table>
<thead>
<tr>
<th></th>
<th>Mg (at.%)</th>
<th>O (at.%)</th>
<th>C (at.%)</th>
<th>P (at.%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>intact layer</td>
<td>4.71</td>
<td>46.05</td>
<td>31.76</td>
<td>6.40</td>
</tr>
<tr>
<td>chipped layer</td>
<td>18.71</td>
<td>57.37</td>
<td>18.39</td>
<td>1.25</td>
</tr>
</tbody>
</table>

3 Conclusion

The study has shown that the photometrical measurement is an adequate method to get information about the degradation of magnesium alloys. The high resolution makes it possible to predict the behavior of magnesium alloys in a body fluid, like plasma. However, the photometrical measuring provides meaningful results. This allows concluding, that the time-dependent data are valid. This enables the prediction of the behavior of different alloys in plasma. The next step is the evaluation of the qualification of presented immersion experiment to forecast degradation in an in vivo experiment.

4 References

[8] F. Evertz, B. Glasmacher; A dynamic in vitro degradation system for standardized Mg degradation studies; Magnesium-Based Biodegradable Implants Symposium; The Minerals, Metals and Materials Society, Annual Meeting 2013, San Antonio
Flocking technology for 3D textile scaffolds
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Introduction
Textiles, especially nonwovens, can be used well as scaffold materials for tissue engineering (TE). Nonwovens can be produced in multiple ways and therefore beneficial properties as well as deficits have to be kept in mind to design an optimal scaffold. The two most prominent types of nonwovens for TE are electrospun nonwovens and staple fibre nonwovens. Electrospun nonwovens provide very fine fibres and can be produced in complex shapes, but hinder cell migration. Staple fibre nonwovens are made of less fine fibres and allow for good cell migration, but cannot be put in complex shape easily.

Methods
Therefore a process based on flocking technology was developed to overcome these deficits. The flocking process was evaluated using a systematic design of experiment setup. In this process fibres are cut into short segments (~4 mm) and deposited onto a gelatine coated mandrel using a strong electric field. The fibres are bonded thermally and the gelatine is washed out again. Finally you get an all textile nonwoven scaffold at complex shapes.

Results
In our experiments proper process parameters to flock fibres onto a substrate coated with gelatine were found. Furthermore we were also able to process thinner fibres than those mentioned in literature before. Thermal bonding as well as mechanical strength without gelatine suffered from a fibre separation during the flocking process.

Conclusion
Flocking technology is a promising approach to produce 3D textile scaffolds and to overcome some deficits of electrospun nonwovens and staple fibre nonwovens. Further research have to be done on thermal bonding as well as adjusting the scaffold properties to the tissues needs.
Influence of fiber diameter on the hemocompatibility of small-diameter 3-layer electrospun vascular grafts

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Abstract

Testing the hemocompatibility of small diameter electrospun vascular grafts represents a key point in their development. Although there have been a few in vitro and in vivo studies regarding their hemocompatibility, all of the in vitro studies have been done under static conditions. In this study, a dynamic hemocompatibility test setup consistent with the properties of 3-layered electrospun grafts has been developed. With this setup, not only has the hemocompatibility of those grafts been tested, but also the influence of the fiber diameter on their hemocompatibility. Thus, two groups of vascular grafts with different fiber diameters, 1.3 µm and 1.7 µm, were tested. The results showed negligible rates of hemolysis induced by the grafts for both fiber diameters. Only one of the two platelet-counting methods employed in this study showed that the fiber diameter of the vascular graft affects their thrombogenicity. This result was statistically confirmed with a significance of p < 0.1. Subsequently, a qualitative scanning electron microscopy (SEM) analysis was carried out on the tested grafts. The SEM analysis showed no differences in the type of platelet aggregations and fibrin mesh between the two graft groups.

1 Introduction

With more than 4 million deaths each year, cardiovascular diseases (CVD) represent the main cause of death in Europe [1]. The need of bypass surgery has therefore drastically increased. In Germany, 97,000 bypass surgeries were performed in 2011 [1]. Using autologous grafts in cardiovascular replacements is not always possible due to the poly-morbidity of CVD patients and the difficulty of finding suitable veins [2]. To overcome this shortage, two types of synthetic vascular grafts are widely used: expanded Poytetra-fluorethylene (ePTFE) and Polyethylene terephalate (PET) which are showing satisfactory results in replacing big diameter vessels [3]. However, these prostheses fail in replacing small diameter (<6 mm) vessels [4]. With a smaller diameter, the patency of these synthetic grafts decreases drastically due to the higher risk of thrombosis. After only 6 months, the patency of PTFE grafts used for a femorotibial bypass sinks to 60% and to 30% after 2 years [4]. To overcome this restriction, tissue engineering is used in combination with electrospinning techniques to create tissue engineered vascular grafts. These grafts, electrospun from a variety of biocompatible and biodegradable polymers, represent a 3-dimensional structure that allows the culture and growth of the patients own endothelial cells. Thus, a high hemocompatibility can be reached creating a biological contact surface between the flowing blood and the synthetic graft.

Taking into account the risk of an incomplete cell growth on the total surface of the grafts or the damage of the cell layer during the implantation, it is important to test the hemocompatibility of those grafts in case of direct contact of the polymer fibers with blood.

Various in vitro studies have been carried out on the hemocompatibility of electrospun vascular grafts fabricated from different polymers. Those studies have been focused on the static interaction between blood components and the graft materials. However, no study has investigated the in vitro hemocompatibility of those grafts under dynamic conditions simulating physiological blood flow conditions.

At the Institute of Multiphase Processes, 3-layered electrospun vascular grafts are spun from polycaprolactone (PCL), Poly lactic acid (PLA) and polyethylene glycol (PEG). These materials are known for their biocompatibility and availability. This study has as main purpose the determination of the influence of fiber diameter on the hemocompatibility of PCL/PLA/PEG 3-layered electrospun grafts. As confirmed by a study of MILLERET et al., the fiber diameter of electrospun scaffolds influences the platelet adhesion as well as the activation of platelets and the coagulation cascade [5]. Due to their porous structure, testing these graft in a water bath under dynamic and physiological conditions poses a leakage risk in the testing system. For this purpose, a test component was developed to ensure an insulation of the grafts.

2 Material and methods

2.1 Polymers

ε-polycaprolactone with a molecular weight between 70,000 and 90,000 Mn was purchased from Aldrich Chemistry. Poly (lactic acid) and poly (ethylene glycol) with 4’000 Mn were purchased from Fluka Biochemica (Steinheim, Germany). The solvent, 2,2,2-trifluorethanol was purchased from ABCR GmbH (Karlsruhe, Germany).
2.2 Electrospinning

The electrospinning of the grafts was performed with an apparatus developed in the Institute for Multiphase Processes. Sterican® hollow needles (B.Braun) with 0.8 mm and 0.4 mm diameter were used. The mandrel collector had a diameter of 4 mm and was rotated with a frequency of 1000 rpm. The applied voltage and the flow rate of the polymer were set at 20 kV and 3 ml/h respectively. The inner and the outer layers were spun from a PCL/PLA/PEG blend, the middle layer was spun from PCL. Each polymer layer was spun for 3 minutes. A camera was installed to observe the process.

2.3 Dynamic hemocompatibility setup

In this study, a modified CHANDLER LOOP system was used. With this system, the tubes can rotate in a water bath which is connected to a temperature control unit as shown in fig. 1.

![Figure 1](image1.png)

**Figure 1** Schematic representation of in vitro modified CHANDLER LOOP system with a temperature control unit

For this study Tygon (TYGON® S-50-HL, SAINT GO- BAIN) tubes were used with an inner diameter of 3.97 mm and a length of 600 mm. To connect the tubes with the grafts, titanium connectors were used. The temperature was set at 37 °C and the tubes rotating speed was 20 rpm which represents a flow velocity of 200 mm/s. A surface to volume ratio of 1/25 was taken for the test. Accordingly 3 ml blood volume was tested in each tube. The test duration was 1 hour.

To realize the testing in the water bath despite the porosity of the grafts, an insulation chamber was developed (Fig.2). In this chamber the grafts can be placed and insulated within a silicon canal.

![Figure 2](image2.png)

**Figure 2** (A) Developed insulation chamber. (B) Silicon being injected in the canal

2.4 Hemolysis, platelet count and platelet adhesion

To investigate the hemocompatibility of the grafts, the different components of the in vitro setup were separately tested. Thus, two groups were tested - one with tubes and one with tubes and connectors.

Hemolysis was determined using photometry. Platelet count was done using two methods: a manual count using light microscopy and an automatic count using an hematology system (Advia® 120, Siemens). The index of hemolysis and the platelet index were determined using the formulas below.

After the test a chemical cell fixation followed by freeze-drying was done to allow a SEM analysis of platelet adhesion.

\[
IH = \left(1 - \frac{HCT}{100}\right) \times \frac{PHb}{GHB} \times 100\%
\]

- **IH**: Index of hemolysis [%]
- **HCT**: Hematocrit [-]
- **PHb**: Plasma hemoglobin [g/dl]
- **GHB**: Total hemoglobin [g/dl]

\[
PI = \frac{PT}{PN} \times 100\%
\]

- **PI**: Platelet index [%]
- **PN**: Platelet count of negative control [1/µl]
- **PT**: Platelet count of tested sample [1/µl]

3 Results

3.1 Hemolysis

The different system components and the grafts do not tend to induce hemolysis. All of the indexes are negligible and below the 0.4% limit set by HAYCOX and RATNER [6]. The highest rate, 0.24%, is induced by the grafts with 1.7 µm fiber diameter (Fig. 3).
3.2 Platelet index

The platelet index based on the results of the Advia hematology system shows that the number of platelets in the blood was reduced after being in contact with the grafts compared with the index of the tubes with connectors. A decrease of 15% was measured for the grafts with a fiber diameter of 1.3 µm and 13% for the grafts with a fiber diameter of 1.7 µm. The difference is confirmed with a significance of $p < 0.05$ for grafts with a fiber diameter of 1.3 µm and $p < 0.1$ for the grafts with a fiber diameter of 1.7 µm (Fig. 4).

The results of the manual platelet counting showed similar tendencies, the platelet index of both groups of grafts was lower when compared with the index of the tube with connectors. This reduction represents 34% for the group of 1.3 µm and was confirmed with a significance of $p < 0.001$. For the group of 1.7 µm the 20% reduction shows no significance.

Comparing the platelet index of both groups of grafts, the results of the manual counting method showed a difference of 13%, which was confirmed with a significance of $p < 0.1$ (Fig. 5).

3.3 Scanning electron microscopy

Different types of platelet aggregations were observed. The SEM analysis showed that the types of platelet aggregations and fibrin mesh between the two groups of grafts were similar. Fig. 6 (a) shows platelet aggregations without the presence of fibrin mesh. In the fig. 6 (b), the adhesion of activated platelets with their pseudopodia on the polymer fibers of the graft can be observed.

(A) Platelet and erythrocyte aggregation within the polymer fiber mesh. (B) Platelets attached with their pseudopodia on the polymer fibers.
Other types of aggregations were observed, for example only fibrin mesh was present (fig. 7 a). In the fig. 7 (b), an aggregation of platelets forming a network with a wide fibrin mesh can be seen.

**Figure 7** Scanning electron microscopy. (A) Fibrin mesh without presence of platelets. (B) Fibrin mesh with presence of platelets.

4 Conclusion

The aim of this study was to realize a dynamic *in vitro* hemocompatibility testing of electrospun vascular grafts. Furthermore the influence of the fiber diameter on the hemocompatibility of electrospun vascular grafts should be investigated. Therefore an insulation chamber was developed to ensure the testing of these grafts with a modified CHANDLER LOOP.

The tested grafts showed a negligible rate of hemolysis of about 0.2 %. These rates are below the 0.4 % limit set by HAYCOX and RATNER [6]. Regarding the platelet index the results have shown a significant decrease of the index after contact between the blood and the grafts. This decrease represents the number of platelets that have built aggregations, have been activated or destroyed when coming in contact with the polymer fibers of the grafts. The results demonstrate that the used test setup in combination with the developed component for testing electrospun vascular grafts is appropriated for dynamic *in vitro* hemocompatibility testing.

The influence of the fiber diameter on the hemocompatibility of the electrospun grafts has been confirmed only with the manual platelet counting method. The counting method using the ADVIA hematology system showed no difference between the two fiber diameters. The qualitative SEM analysis has shown no differences between the two groups in the type and the dimension of the platelet aggregation within the polymer fibers.

4 References


Polymer-based, biodegradable, brain-derived neurotrophic factor-containing coatings on cochlear implant electrode carriers for enhancement of spiral ganglion cell growth - investigation of drug load and degradation behaviour

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Abstract

With the purpose to enhance spiral ganglion cell growth after cochlear implant insertion in order to preserve the residual hearing, we propose a polymer-based local drug delivery system for growth factor release. Therefore, the biodegradable natural poly(4-hydroxybutyrate) and the synthetic poly(L-lactide) were used as coatings matrices on the silicone-based electrode carrier material, and different surface activations were performed in order to enhance the effectiveness of surface attachment of brain-derived neurotrophic factor (BDNF) via absorption. Relating to this system it was shown that activation of the surface leads to an increased adsorption of BDNF. Furthermore, an in vitro degradation study of the used polymer matrices P(4HB) and PLLA under quasi-stationary conditions in human perilymph supplemented with enzymes was performed.

1 Introduction

Cochlear implants (CI) are well accepted for the effective treatment of sensory deafness. These implants include an electrode array that directly stimulates the cochlear nerve, by bypassing the damaged hair cells. An important point is the maintenance of the residual intact spiral ganglion cells and therefore the rest hearing [1]. With the purpose to enhance the spiral ganglion cell growth, a specific CI surface modification enabling the release of nerve growth factors like brain-derived neurotrophic factor (BDNF) would be desirable [2]. However, this requires a biofunctionalization method, allowing for sustained BDNF release from CI surfaces over time. This could be provided by poly(4-hydroxybutyrate) (P(4HB)) or poly(L-lactide) (PLLA) coatings. The present study examines the feasibility of BDNF-loading on unmodified and activated P(4HB), PLLA and silicone. Furthermore, the study addresses in vitro degradation behaviour of the polyesters P(4HB) and PLLA. The degradation of the polymers was performed under quasi-stationary conditions, using human perilymph supplemented with enzymes contained in the human cochlea.

2 Methods

Biodegradable polymeric films were spray-coated onto the surface of silicone films (Ø = 6 mm), which acted as model samples of the silicone-based CI carrier [3]. On the polymeric P(4HB) or PLLA surfaces reactive groups were generated via: a) O2 plasma, b) NH3 plasma, c) O2 plasma and subsequent reaction with (3-aminopropyl)triethoxysilane (APTES) (5% (v) in water).

For adsorption of BDNF the activated and non-activated polymer-coated silicone films were transferred into a BDNF solution (c = 1 µg/mL in phosphate-buffered saline (PBS) pH 7.6) for 16 h at 4°C. In order to mimic the conditions of the inner ear a quasi-stationary release model (non-sink conditions) [3] was used to release BDNF in artificial perilymph pH 7.3 (formulation according to [4]). Determination of obtained BDNF surface loads and in vitro release was carried out by using an enzyme-linked immunosorbent assay (ELISA), using a RayBio Human BDNF ELISA Kit.

For degradation studies test specimens of 10x5 mm of P(4HB)- and PLLA-films were fabricated [5] and the quasi-stationary degradation model (non-sink conditions, 37°C in artificial perilymph) [3] was used. To the artificial perilymph (pH 7.3) the enzymes lactate dehydrogenase (LDH) and butyrylcholinesterase (But) (45 mU/mL LDH-5, 8 U/mL But, 0.5 mM NADH) were added. The molar mass loss of P(4HB) and PLLA over time was analyzed at 30°C using a PSS SECcurity SEC system (Polymer Standard Services GmbH, Mainz, Germany). Separation was performed with three PSS SDV columns (10³, 10⁵ and 10⁶ Å respectively). Chloroform was used as the eluent at a flow rate of 1 mL/min [6].

3 Results

Differences of BDNF surface loads between polymeric coating materials and the various applied activation methods could be observed. Looking at the untreated surfaces, PLLA revealed the highest BDNF surface load compared to P(4HB) and silicone.

Both biodegradable polymeric films show an increased BDNF surface load after NH3 plasma activation and an even higher load after modification with APTES. Treatment with O2 plasma led to a decreased adsorption of BDNF at PLLA and silicone surfaces.
Through generation of functional groups, BDNF surface loads could be improved with the highest adsorption on the APTES-activated PLLA surfaces (image 1).

![Image 1 BDNF surface loads on differently activated silicone films and with PLLA and P(4HB) spray-coated silicone surfaces, each with n=3 samples.]

A previous shown the release of BDNF, adsorbed to an untreated P(4HB) surface, could be observed [7]. Due to the fact that general drug release from biodegradable polymers can be affected by degradation, in vitro degradation studies for both polymers (P(4HB) and PLLA) were investigated. PLLA with a molar mass of \( M_w = 660,000 \) g/mol shows a nearly linear molar mass loss during the observed time interval while P(4HB) (\( M_w = 165,000 \) g/mol) has a slightly accelerated degradation until about 8 weeks, after that an equally linear reduction of the molar mass occurred. At the terminal examination (52 weeks), a very low average molar mass of \( M_w = 16,000 \) g/mol (about 10% of initial molar mass) could be found for P(4HB), whereas PLLA still had a molar mass of about \( M_w = 200,000 \) g/mol (about 30% of initial molar mass), evidencing its slower degradation behaviour (image 2).

![Image 2 Molar mass loss of P(4HB) and PLLA films over 52 weeks, each with n=5 samples.]

4 Conclusion

It could be shown, that adsorption of the growth factor BDNF to the polymeric coatings of silicone foils is possible. In majority of cases, generation of reactive groups to the polymeric surface enhances the BDNF surface attachment. We observed for all analysed polymers good results using the surface activation with APTES. P(4HB) has a higher degradation rate in vivo than in vitro. Enzymes accelerate the in vitro degradation and the hydrolytic ester cleavage becomes less important [8]. PLLA shows comparable degradation rates in vivo and in vitro [8], even though an increased enzyme activity can be seen in close proximity of the implant [9]. Neither in vitro nor in vivo studies of these polymers in a stationary system similar to human cochlear were shown in the literature. The in vitro degradation study of the polymers P(4HB) and PLLA revealed a relatively fast degradation for both polymers. Importantly, the degradation studies were performed taking into consideration the physiological conditions of the inner ear.

In this preliminary study we successfully demonstrate a biodegradable polymeric local BDNF delivery system (P(4HB) or PLLA) for silicone based cochlear implant electrode carriers.

5 Acknowledgement

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6 References


Protective waistcoat for goats in a long-term animal model

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Abstract

Drivelines for ventricular assist devices and catheters for peritoneal dialysis are percutaneous implants which are designed for a dwell time of at least one year. The testing of such percutaneous devices in animal experiments should represent this long period, since infection episodes can occur a long time after implantation. Usually, these animal experiments are the final step in the previous development of the implant and represent a high monetary value. However, a single malfunction is already sufficient to end the experiment. As part of a research project preclinical testing of percutaneous implants was performed in goats. Although the percutaneous devices do not cause pain, the animals will bite and pull at them and endanger the experiment. Therefore, protection of the implant is required. Standard wound dressing is not sufficient and appropriate protective garments for goats are not commercially available. Therefore, custom fit protective waistcoats were designed and manufactured. To this end the size of the goats was measured and a pattern for sewing was created. A polyester mesh fabric provides the necessary resistance and good breathability. The waistcoats were reinforced with polyethylene foam to prevent biting through the garment. Side-release buckles were chosen as fasteners. A total of six waistcoats were manufactured. They fit the goats tightly, while preserving the full range of motion. The goats tolerated them well. They are durable, secure and effectively protected the implants for a period of over one year.

1 Introduction

Animal experiments are preceded by long and laborious technical work in the laboratory, and usually a large investment has been made in its development. In the case of drivelines for ventricular assist devices (VAD) and catheters for peritoneal dialysis (PD) the devices are designed for a dwell time of at least one year up to several years. The duration of the animal experiments has to represent the functional time of the devices. Zierer et al. reports driveline infections in patients receiving assistance by VADs in 23% of the cases and late-onset infection episodes as late as 7 months after the implantation [1]. As part of a research project, a newly developed infection-resistant percutaneous device for drivelines for ventricular assist devices or catheters was to be tested in an animal experiment. The test device consists of a catheter which is equipped with an extricable membrane to prevent infections [2,3]. The percutaneous device is intended to last for a period of two years. As an animal model the goat was chosen. The objective of the animal test is to prove the effectiveness of the implant, which is to remain free of infections. In each animal, two devices were implanted percutaneously. They do not cause pain to the animal but they might itch, however, and be perceived as a foreign object and such causes the animal to try to remove it. The goat’s natural behavior shall not be compromised, which means they are not restrained or fixed to the cage, but can run and jump around freely, fight against each other and graze outside. During the long experimental period, the animal has a vast amount of time to explore, nibble, rub and scratch. But the project is put at risk if the animals manipulate or damage the catheters. Consequently, it is crucial for the success of the experiments to thoroughly shield off the catheters and the implantation site. In addition, long-term animal experiments are linked to long-lasting and resource- and cost-intensive preparatory work and no unnecessary harm and restraining must be done to the animals. It is therefore of major importance to equip the animals with proper protective garments. This need was already recognized many years ago by Vatner et al. who described backpacks for telemetry equipment for baboons, dogs, and goats [4]. Today, several companies provide protective equipment for primates, pigs, dogs, and rodents. Appropriate protective waistcoats for goats were not commercially available and the use of goats for pigs and dogs failed. Therefore, protective waistcoats for goats had to be designed and manufactured. They should be inexpensive, easy to make, and deliver a safe and effective shielding of the implant from any adverse actions by the goat in a long-term experiment.
2 Animals, Materials and Methods

2.1 Choice of Animal

Goats are widely used as laboratory animals in various fields of research [5-11]. In the work presented here, the goat (German improved white goat - capra aegagrus hircus) was chosen as the animal model, because it offers a human-comparable body for real-sized implants. Also, it is generally good-natured and can be trained [5,12]. Its skin tolerates percutaneous implants well. Unfortunately, goats have a strong oral investigative need [13]. Wounds, sutures, percutaneously implanted and external devices are put at risk by licking, biting and tugging. In long time experiments the change of pelage can raise additional problems.

2.2 Requirements

The protective waistcoats should meet the following set of requirements: Protection of the catheters against biting, pulling and scratching, preservation of the goats’ full range of motion, high breathability to prevent heat build-up, durability, easy cleaning and manageability, resistance against disinfecting cleaning agents, quick access to implantation sites, easy manufacturing, and low price.

2.3 Size Determination

The protective property of the waistcoat depends mainly on two factors: the right cut of the garment and the material. The area to be protected has to be fully covered plus an additional safety margin. The dotted line in Figure 1 indicates the area to be protected.

Figure 1: Measurement points: Lengths (A-C) and circumferences (1-6). The implantation area is indicated by the dotted line.

A set of measurement points was acquired from the live animal (dashed lines in Figure 1). These measurements were used to create a paper pattern (Figure 2). The lengths are labeled A - C, and circumferences 1 - 6 (see Figure 1).

The ventral length consists of the values A and B. The circumference is reduced at the cranial and at the caudal end (Part A and C in Figure 1) to prevent the garment to slip out of position. The distances between the circumference measurements (2), (3) and (4) in Figure 1 were added to the data set to determine the position of the measured circumferences. A sample data set of the measured values of two goats is shown in Table 1.

<table>
<thead>
<tr>
<th>ID</th>
<th>Circumference (cm)</th>
<th>Length (cm)</th>
<th>Distance (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Z94</td>
<td>47 80 88 97 89 82</td>
<td>10 60 15</td>
<td>18 35</td>
</tr>
<tr>
<td>Z95</td>
<td>50 80 92 97 88 82</td>
<td>10 60 10</td>
<td>20 35</td>
</tr>
</tbody>
</table>

Table 1: Sample measurements for two different goats

The two elliptical leg openings are 140x180 mm wide. Their centers are 210 mm from each other and 150 mm to measurement point (2). To achieve the reduction in circumference around the neck, a dart is inserted in the center of the cranial end.

Freedom of movement of the hind legs is ensured by shortening the ventral part accordingly. Owing to the caudal location of the implantation site, the waistcoat is designed in an asymmetrical shape to fully cover the implantation site. In Figure 2 the dotted lines indicate the symmetrical cut. By moving points (3) and (6) 5 cm and (4) and (5) 10 cm to the left, the resulting asymmetrical cut is achieved.

Figure 2: Pattern for the protective waistcoat. Dashed lines indicate the location of the measurements, thick dashed lines the foam inset. The dotted lines represent the symmetrical, the solid line the asymmetrical version.

To prevent the goat from biting through the garment a sheet of foam is inserted in a pocket at the inside of the waistcoat (indicated by the thick dashed line in Figure 2). The pocket is closed with a velcro fastener, so the foam sheet can be removed from the pocket for cleaning.

An additional 10 - 20 cm should be added to the circumferences (4) and (5) for an underlapping flap at the dorsal opening area. This is to prevent the goat from reaching underneath the waistcoat near the closure. As closure mecha-
nism for the waistcoat side release buckles are attached in such a way that waistcoat can overlap when it is fully closed. The ends are held in place by velcro fasteners.

2.4 Selection of Materials
A 3D mesh fabric made of polyester with a thickness of 2 - 3 mm was chosen for the outer shell to achieve the necessary resistance against chewing (saliva), heavy tugging and good breathability (Hüco Stoffe, Berlin, Germany, article number K22022/6, 270 gm²). A thin polyester sport mesh fabric was chosen for the pocket on the inside of the waistcoat (Hüco Stoffe, Berlin, Germany, article number K4007, 200 g m⁻²). The fabrics can be sewn with a household sewing machine using commercially available polyester yarn. They are also easy to clean: They are washable at 60° C in a standard washing machine, resistant against disinfecting cleaners and can be tumble dried.

To keep the animals from grabbing and moving objects, such as catheters, underneath the fabric, a 10 mm thick and 250x350 mm large piece of polyethylene foam is included in the waistcoat directly above and around the implantation area (Figure 4). It is soft enough to adapt to the goats’ body shape but prevents biting through the waistcoat. Also it gives stability to the caudal end part of the waistcoat. The foam can be slipped into a pocket on the inside of the waistcoat.

Eight single adjustment side-release buckles are chosen as the closure mechanism, since goats manage to open the velcro fasteners and to break or rip off buttons. Zip fasteners offer the most snug and durable closure, but size adaptation is not possible. The side-release buckles allow a change in size and easy handling for the caretakers.

2.5 Sewing of the Waistcoats
After transferring the pattern to the fabric, adding a seam allowance and cutting it, the waistcoats are sewn with a household sewing machine (Textima Veritas 8014/43) using standard polyester yarn (NM 80) and a standard multipurpose needle (130/705H size 90). The double lock stitch is used for all seams. The leg openings are covered with a 25 mm bias binding and a double seam to prevent ripping. A seam is made around the complete waistcoat. Then, the underlap is cut and seamed and equipped with the velcro fasteners. The opposite parts are attached to the main piece cross-wise and the underlap is sewn to the main piece. This seam is reinforced with a second seam. Bar tacks are used to attach the side-release buckles to the garment.

2.6 Animal Experiments
The two goats were kept together in a pen at the Department of Experimental Medicine, Charité – Universitätsmedizin Berlin and later at BLS Preclinical Services, Berlin. Humane care and use of the goats was assured. Only legal procedures were used (governmental permission G 0324/09). Two weeks before surgery, the goats adapted to wearing the protective waistcoats. They tolerated them well, so that after three days of short training periods the goats wore the waistcoats continuously.

In each animal one test catheter and one control catheter were implanted parallel to each other on the left side behind the costal arch. The waistcoats were put on immediately after surgery. Besides checking the animals for general well being on a daily basis, the implants were checked one to two times per week. For this purpose, three to four of the side-release buckles at the caudal end were opened so that the implantation site could be accessed. After examining the implants and changing the dressings, the waistcoat was closed and checked for any damages.

3 Results
Three waistcoats for each of the two goats were made according to the pattern. They fit the goats well and preserve their full range of motion (Figure 3). The goats tolerated the waistcoats well. The waistcoats were used for a period of more than one year and effectively protected the implants from the animals' attempted mutilations. The garments withstood the goats’ everyday behavior: Biting, tugging, scraping, using hooves, snouts and horns, and rubbing against walls. The foam inset (Figure 4) effectively prevented biting through the garment.

The side-release buckles resisted the stress of biting and scratching by the animals. However, over time some - 5 out of 50 - buckles failed and had to be exchanged. The belts on the adjustable parts of the buckles were affixed with adhesive tape to prevent accidental tightening by the goats’ tugging on the belts. The waistcoats were changed for cleaning at least once every two weeks or more often when necessary. The foam insets were removed and cleaned separately. The garments were machine washed at 60°C and tumble dried. After many months of use some wear is visible, but no structural damage occurred.
4 Conclusion

The waistcoats kept the goats from mutilating the implants and maintained their full range of motion at the same time. Using the waistcoats to protect the implants made it possible to have the animals running around freely.

The use of the side-release buckles proved advantageous: The goats are not able to open the fasteners, but for humans it is possible to open or close the fasteners with only one hand. In addition, the waistcoats are adjustable in diameter, which makes it possible to use the waistcoats over a long period of time, even when the goats grow or gain weight.

It is unknown if the failure of some of the buckles was caused by the goats or by the washing machine. The lack of one side-release buckle did not decrease the protective function, but the use of metal buckles is recommended.

One of the implants was inserted at a very caudal position. Therefore, the waistcoats had to be longer than initially planned. Only thanks to the foam inset could enough stability be provided.

The addition of a pocket for the foam inset made it possible to remove it for cleaning. This way, the waistcoat can be washed in a washing machine, which facilitates cleaning.

Compared to commercially available protective garments, the work presented here turns out to be very inexpensive. The expenses for materials (fabric and accessories) per waistcoat amount to only €25. If the work is commissioned to a professional tailor, the costs are estimated to below €100 altogether.

Very durable and effective protective waistcoats could be made, on short notice and at a low price, to ensure the successful completion of long term animal experiments with percutaneous implants.

The presented work was funded by the German Federal Ministry of Education and Research (grant number 13EZ0855). The authors would like to thank Nadine Banfi, Moritz Ringelstein, Ludwig Zabel and Kathrin Schneider for their assistance and advice.

5 References

Cross section studies of laser-structured electrodes with locally adapted thicknesses

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Introduction

We introduce a novel picosecond (ps) laser-structured electrode array with locally adapted thicknesses. Using a LUMERA LASER Rapid10 ps laser, MP35N metal tracks were thinned down locally from 25 µm to approximately 5 µm on a 60 µm thick silicon rubber substrate while the electrode sites’ thickness remained 25 µm. Applying a 20 µm thick top layer insulation of silicon rubber the resulting surface of the array is planar after opening the electrode sites by laser structuring.

Gaining information about the interlayer’s properties is crucial for the comparison of the arrays with those from other technologies (e.g. thin-film polyimide based arrays). Due to the elastic properties of silicone rubber, the common cross-section preparation by polishing is not feasible. Freezing induced cross sections are the standard for these examinations.

Methods

Some of the electrodes were frozen at -196 °C using liquid nitrogen. Cross sections were obtained by breaking the frozen samples.

The remaining electrodes were laser cut with the LUMERA LASER Rapid10 in a straight line through specific points. Afterwards the samples were fixated for further investigation with a light microscope. Using a calibrated microscope the layer thicknesses could be measured and the structural quality of the electrode array be assessed.

Results

It was possible to obtain comparable cross sections with both methods. However, it was much easier to obtain cuts at specific points with the laser. While the preparation time is shorter with the laser, these samples have to be cleaned with ethanol and a microbrush prior to inspection.

Conclusion

Preparation of samples through ps laser cutting as an alternative method for cross-sections in silicone rubber electrodes arrays was established.

Freezing induced cross sections might still offer a preferable breaking edge for electronmicroscopy. However, ps laser cutting delivers good quality cross-sections for light microscopy.
Design and evaluation of an inertial flow apparatus for dynamic blood-compatibility testing

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Abstract

In vitro-hemocompatibility testing is commonly used for parameter studies on e.g. cardiovascular implants or materials for blood-ducting tubing. Two basic working principles for dynamic in vitro-testing have found widespread use: Circuits with roller pump drive and CHANDLER-type apparatus. Both have specific disadvantages. To enhance testing conditions, a new in vitro-test device designed to avoid potentially harmful shear stress and blood-air-contact was constructed and evaluated. Its working principle – an oscillating motion transformed to unidirectional flow by means of an unidirectional restrictor valve - is based on works of WOLF and VAN OEVEREN [1],[2]. Nine different disposable valves from seven manufacturers were evaluated with regard to flow resistance. An adjustable oscillation shaker is used to drive a specimen holder in a housed waterbath. The new system’s performance was evaluated for two different valves and porcine whole blood in comparison to a CHANDLER-loop setup. Results show that, at similar wall shear stress, the valves have no adverse effect on background activation and hemolysis, while oxygen saturation is decreased. The new inertial flow device provides a way to avoid blood-air-contact during experiments while allowing simultaneous testing of 18 samples with defined pulsatile flow.

1 Introduction

Common in vitro blood compatibility testing devices are only partially capable of emulating blood's physiological environment. In vitro-systems driven by roller pumps allow for a wide range of flow rates and can be used to emulate pulsatile blood flow. However, pumping introduces mechanical stress on blood components, increasing system-related blood damage. Another common class of testing devices are the CHANDLER-like apparatus that make use of gravity instead of an external pumping mechanism to generate blood flow through closed tube rings. The working principle requires that tube rings are only partially filled with blood. This leads to a blood-air-interface within the test system which contradicts physiological conditions. Both alternatives cause increased activation of blood components resulting in limited reproducibility of experiments with whole blood. The aim of the presented Bachelor’s thesis was to design and validate a system suitable to conduct dynamic haemocompatibility experiments while exposing blood neither to high mechanical forces nor excessive air contact. One feasible solution is a valve-driven in vitro-system, as described by WOLF and VAN OEVEREN [1],[2].

2 Design considerations

A valve driven in vitro-test system or inertial flow apparatus (IFA) has three main components: Tubing, motion control, and unidirectional restrictor valves. Tygon®S50HL (SAINT GOBAIN PERFORMANCE PLASTIC) was chosen as tubing-material because of low background activation, availability and extensive comparative data from past experiments. Various concepts were discussed for the realization of a drive unit and the simplest, most effective solution was selected. The drive unit consists of an electric motor with adjustable speed driving a flywheel. An eccentrically mounted rod is connected to a shaft, thus resulting in an oscillating shaft-motion. The specimen holder can either be directly attached to shaft or motion-coupled by two connected excenter tappets. The system was adapted to an existing temperature-controlled water bath. Therefore, the specimen holder and oscillating shaft were fitted to appropriate spacial dimensions. Main focus of development was specimen's fast interchangeability to grant for fast further specimen processing. An additional requirement was scalability – the construction is designed to test a variable number of samples (up to 18) simultaneously [Fig. 1].

Fig. 1: Setup with drive unit and specimen holders immersed in a temperature-controlled water bath.
3 Methods

Flow characterization experiments were performed with a model-fluid consisting of water and glycerine adjusted to a dynamic viscosity of 4 mPas, which equals blood’s viscosity at approximately 50 % hematocrit. The blend’s density was determined to 1.1 g/cm³. An initial evaluation included nine disposable valves from seven manufacturers. Two valves that exhibited least flow resistance to gravity flow and thus maximum volume flow at a given pressure interval of 0.03 bar were chosen for further testing (BECTON DICKINSON GMBH/valve A & PIEPER FILTER GMBH/valve B). One main parameter for recreating flow conditions of various blood vessels is mean volume flow. Resulting mean volume flow was measured for four different rotational speeds. A specimen holder was attached to the oscillating shaft, 300 mm tubing attached to the specimen holder and a valve was mounted at the tubing’s end. Rotational speed was measured with a laser revolution counter at the flywheel. Volume flow is determined measuring output fluid mass. Results for both valve types are shown in Figure 2.

![Graph showing volume flow through 300 mm tubing and valve at different speeds. Key: ■ valve A; × valve B](image)

Additionally, background activation was compared for the new IFA versus a CHANDLER-loop system. Wall shear stress was selected as a suitable parameter for hydrodynamic comparison [3]. Mean wall stresses of both systems were equalized to 1.15 Pa which corresponds to 10 rpm (CHANDLER-loop) resp. 135 rpm (IFA). The IFA was evaluated using valves A and B. For hemocompatibility evaluation, fresh venous porcine whole-blood was used. Hemocompatibility evaluation was conducted by platelet count and rate of hemolysis. Hematocrit served as a control.

4 Results

Platelet count does not differ significantly comparing the IFA with valves A and B and the CHANDLER-loop [Fig 3].

![Graph showing normalized platelet count for CHANDLER-loop (n=12), IFA /A(n=12), IFA /B (n=4)](image)

Hemolysis however showed a slightly positive trend in favour of the IFA for valve A [Fig. 4].

![Graph showing normalized hemolysis for CHANDLER-loop (n=12), IFA /A(n=12), IFA /B (n=4)](image)

Additionally, a change in the blood’s colour was observed for test runs with the IFA. Blood from IFA CHANDLER-loop tubing was noticeable brighter than blood from the IFA. The darker color in the latter is due to a decrease in oxygen saturation from cellular metabolism. This effect is not observed in the CHANDLER-loop setup, where the blood is kept in an arterial state because of gas exchange with the entrapped air [Fig. 5].

![Image showing blood samples from CHANDLER-loop (left) showing brighter color than from IFA (right)](image)

5 Conclusion

It has been shown that the in vitro-system presented in this Bachelor’s thesis is suitable for regular laboratory use. For compareable wall shear stress, it is operating at...
the same background activation levels as the routinely deployed CHANDLER-loop apparatus, while in contrast providing pulsatile flow with no air present in the tuberings. Gradual oxygen depletion of the blood during experiments leads to a color change that may affect photometric measurement. This results in slightly overestimated hemolysis rates for blood samples taken from the IFA. However, this is true for all closed-loop blood conduction devices without oxygenation. Still, further work should include detailed consideration as to how results may be affected by low oxygen levels, particularly when CHANDLER-loops and IFAs are used within the same study.

6 References


Towards the development of a bioartificial lung - Endothelialisation of TiO$_2$ coated oxygenator membranes

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Introduction
The use of extra corporeal membrane oxygenation (ECMO) devices, which is indicated for patients awaiting lung transplantation, is still limited to a few weeks only, due to thrombus formation and deposition of blood components within the device. Therefore, the basic idea is to improve the haemocompatibility by endothelialisation of the poly-4-methyl-1-pentene gas exchange membranes (PMP), which necessitates the development of coating techniques for the mediation of endothelial cell adhesion to the hydrophobic polymer. The pulsed vacuum cathodic arc plasma deposition (PVCAPD) technique has been shown to enable the coating of thermosensitive polymers. Hence the eligibility of Titaniumoxides (TiO$_2$) deposited on PMP using PVCAPD as an effective coating technique for enabling the endothelialisation was assessed.

Methods
Thin PMP foils (3 x 3 cm, 50µm) were coated with TiO$_2$ via PVCAPD and characterized by SEM, EDX and oxygen transfer rate measurement. Umbilical cord blood derived endothelial cells (hCBECs) were seeded on such samples and incubated for 24 h. Established monolayers were investigated for expression of activation-relevant marker genes and subjected to a leucocyte adhesion assay. Flow resistance and self-healing capacity were assessed in a laminar flow chamber applying 30 dyne/cm$^2$ for 24 h.

Results
SEM and EDX analysis confirmed the homogeneous deposition of nanoscalic TiO$_2$ particles, which only negligibly impaired the oxygen permeability. hCBECs exclusively adhered to areas of PMP foils coated with TiO$_2$. Gene expression analysis revealed that hCBECs seeded on the TiO$_2$ coated surface retained the non-activated state, additionally confirmed by a leucocyte adhesion assay. Furthermore, the established monolayer was resistant to high physiologic shear rate. Besides planar PMP, the coating technique was successfully applied to 3D hollow fibres.

Conclusion
This study demonstrated that TiO$_2$ coating via PVCAPD is a promising technique for coating thermo-sensitive PMP gas exchange membranes, enabling the generation of a non-activated and flow-resistant EC monolayer.
Composition and ultrastructure of PDLLA nanofibers with incorporated rhBMP-2 and rhVEGF$_{165}$ as scaffolds for tissue engineering

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Abstract

The objective of the present work is to fabricate and develop growth factor-PDLLA nano-composites by electrospinning into 2D fleece or 3D tubular scaffolds that can mimic the nature and function of the native extracellular matrix of bone tissue or artificial blood vessels. The bioactive growth factors $^{125}$I-rhVEGF$_{165}$, $^{125}$I-rhBMP-2 and the fibrous protein collagen were incorporated directly into the nascent PDLLA fiber during the electrospinning process. The morphology and distribution of the bioactive molecules in the electrospun fibers were investigated using TEM. The micro mechanical deformation was studied in tension tests using ESEM. The electrospun PDLLA nanofibers loaded with rhVEGF$_{165}$ and collagen showed a ductile behavior with necking and cold drawing deformation on stretching. The incorporation of rhVEGF$_{165}$, rhBMP-2 and collagen molecules into the scaffolds of PDLLA nanofibers could enhance cell attachment, reduce the scaffolds lifetime and promote angiogenesis and osteogenesis process, and thereby induce bone formation.

1. Introduction

Mimicking the extracellular matrix (ECM) of bone tissue remains a significant challenge to repair bone loss due to accidents or disease [1]. Electrospun nanofibrous scaffolds are attractive substrates that may mimic the structural organization of the ECM of bone tissue [2]. Besides traditional 2D nanofibrous structures, electrospinning is powerful in fabrication of 3D fibrous macrostructures, especially for tissue engineering applications [3]. The electrospun scaffolds or 2D fleeces with porous structures provide a space for the cells to invade, proliferate and grow [4, 5]. True 3D structures such as tubes may also be made.

Several attempts have been made to mimic the structural, mechanical, and biological behavior of natural bone [6]. Although major progress was made in the field of bone tissue engineering during the years, synthetic bone grafts, still have several limitations due to the lack of angiogenesis and osteogenesis properties [7]. Herein, it will be shown that the three proteins rhBMP-2, rhVEGF$_{165}$ and collagen can be quantitatively incorporated into PDLLA nanofibers produced by electrospinning. The incorporated proteins can be visualized by TEM after OsO$_4$ staining.

2. Materials and Methods

2.1. Electrospinning of PDLLA nanofibers composites with rhVEGF$_{165}$ and rhBMP-2

PDLLA nanofibers and nanocomposites were fabricated using a high voltage power supply (Heinzinger Electronic GmbH, Germany) with a vertical spinning configuration at room temperature [8]. Briefly, 7.5 wt% PDLLA (Resomer® 207 S, Boeringer Ingelheim Germany) dissolved in a mixture of 80 % Hexafluoroisopropanol (HFIP) from Sigma–Aldrich, Germany and 20 % distilled water (H$_2$O). The spinning process was performed at 16–30 kV applied voltage with a flow rate of 0.1ml/hr using an infusion pump (Pilot A2 Syringe Pump, MC Medizintechnik GmbH, Germany). The bioactive proteins (rhVEGF$_{165}$ and rhBMP-2) were obtained from Morphoplant GmbH and their biological activity was determined [9]. For quantification the two proteins were labelled with $^{125}$I as described [10]. The aqueous solutions of rhVEGF$_{165}$ and rhBMP-2 proteins were mixed with PDLLA solution dissolved in HFIP with a final concentration of 7.5 wt % PDLLA. Collagen type I was obtained from Botiss Dental GmbH, Germany. The collagen was then freeze-dried for 24 h before electrospinning.

2.2. Electron microscope

The morphology and size of the electrospun PDLLA nanofibers and their composites with bioactive
molecules were investigated by using a scanning electron microscope (SEM) (JSM 6300 JEOL). In addition, a conventional transmission electron microscope (TEM) (LEO 912 EFTEM, operated at 120 kV) has been used for characterization of the electrospun PDLLA incorporated with rhVEGF165 and rhBMP-2. The in situ tensile test of the electrospun nanocomposite nanofibers were investigated using environmental scanning electron microscope, ESEM (FEI Quanta 650, operated at 10 kV) [11].

3. Results and Discussions

3.1. Incorporation of rhBMP-2 and rhVEGF165 into PDLLA nanofibers.

In Table 1 it is shown that under the present experimental conditions $^{125}$I-rhBMP-2 and $^{125}$I-rhVEGF165 can be incorporated into the electrospun nanofibers in amounts of 1-2 mg/g PDLLA. Similar amounts have been incorporated into PDLLA tablets by supercritical foaming with CO$_2$ [12].

<table>
<thead>
<tr>
<th>Protein</th>
<th>Concentration mg/ml</th>
<th>Incorporation* 125I-mg/g PDLLA</th>
<th>Yield %</th>
</tr>
</thead>
<tbody>
<tr>
<td>$^{125}$I-rhBMP-2</td>
<td>0.6 - 0.9</td>
<td>1.69 ± 0.49 (4)</td>
<td>60-80</td>
</tr>
<tr>
<td>$^{125}$I-rhVEGF165</td>
<td>0.6 - 1.2</td>
<td>1.22 ± 0.057 (2)</td>
<td>60-80</td>
</tr>
</tbody>
</table>

*Mean ± S.E., n = number in parenthesis.

The yield was calculated to 60-80% as compared to the initial amount of the growth factor used in the spinning solution. The negative balance is attributed to the amount of the spinning solution remaining in the syringe.

3.1.1. Ultrastructure of rhBMP-2 Composite

Fig. 1 shows the TEM micrograph of the electrospun PDLLA/rhBMP-2 after chemical staining with OsO$_4$. In contrast to rhBMP-2, the rhVEGF165 molecules were found to be incorporated in discrete islands or particles. The distribution of rhVEGF165 particles (molecular dimensions ca. 6 nm) in the nanofibers is relatively homogenous with diameters of ~ 20 nm possibly also indicating some aggregation. Large linear aggregates as seen with rhBMP-2 were absent.

3.1.2. Ultrastructure of rhVEGF165 Composite

Fig. 2 shows the TEM micrograph of the electrospun PDLLA/rhVEGF165 nanofibers in a fleece after chemical staining with OsO$_4$. The distribution of rhVEGF165 particles (molecular dimensions ca. 6 nm) in the nanofibers is relatively homogenous with diameters of ~ 20 nm possibly also indicating some aggregation. Large linear aggregates as seen with rhBMP-2 were absent.
3.2. Electrospun PDLLA/Collagen (1:1)
Electrospun PDLLA/Collagen (1:1) nano-composite nanofibers were prepared using HFIP as a solvent. The morphology of electrospun PDLLA/Collagen is shown in Fig. 3. The spun PDLLA/Collagen nanofibers have a uniform structure with an average size diameter of ~500 nm.

PDLLA nanofibers, was blended with collagen type I to simulate the nanostructure of natural ECM and to enhance the cell attachment properties of the final fibers [13].

3.3. Micro mechanical deformation of electrospun PDLLA/rhVEGF165 nanofibers
It is very important to understand the mechanical performance of the electrospun PDLLA composites. The micromechanical properties of the as spun PDLLA composite nanofibers scaffolds were evaluated using in situ qualitative tensile testing in the ESEM.

Fig. 4 shows the micrograph of stretched PDLLA/rhVEGF165 fibers. It is shown that stretching induces micro neck formation (constrictions) in the nanofiber composites. The ductile behavior of the electrospun PDLLA/rhVEGF165 is attributed to the increased molecular orientation of the polymer in the nanofiber and is apparently not infringed by rhVEGF165. The high molecular orientation of polymer molecules in the electrospun nanofibers enhances their mechanical properties (e.g., higher elastic modulus and strength) as compared to bulk materials [11].

3.4. Micro mechanical deformation of electrospun PDLLA/Collagen (1:1) nanofibers
The micro mechanical stretching of the electrospun PDLLA/Collagen (1:1) is shown in Fig. 5. The nanocomposite nanofibers reveal a similar mechanical behavior with necking formation and some necking zones as the PDLLA/rhVEGF165 fibers again indicating that the inclusion of proteins in PDLLA nanofibers has little influence on the mechanical behavior.

3.5. 3D Structures of electrospun PDLLA nanofibrous scaffolds
Fig. 6 shows the electrospun PDLLA nanofibers with 3D structure. The tubular structure could be obtained by collecting the nanofibers on rotating Teflon rod for 6 hours, then carefully removing the tubular scaffold.
They might be useful in future designing of resorbable artificial blood vessels loaded with other specific growth factors and collagen could potentially be used in the therapy of circulatory problems.

4. Conclusions

The present work demonstrates the ability to design 2D fleece and 3D tubular nanofibrous scaffolds loaded with bioactive molecules (rhVEGF_{165}, rhBMP-2, and collagen) which might mimic the native ECM of bone tissue. Blending collagen with PDLLA nanofibers may induce the cell attachment. Moreover, rhVEGF_{165} and rhBMP-2 will stimulate the angiogenesis and osteogenesis process to induce bone formation. TEM micrographs of nanofibers in fleece with incorporated growth factors revealed that rhVEGF_{165} is incorporated in discrete islands and rhBMP-2 molecules may fuse to large linear arrangements. The micromechanical investigations of the nanocomposite nanofibers revealed that they have a ductile behaviour on stretching with good mechanical properties. The combination of rhVEGF_{165}, rhBMP-2 and collagen with PDLLA nanofibers scaffolds with different 3D structures can be used as scaffolds for bone tissue regeneration.

5. References


6. Acknowledgments

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Release dynamics and biological activity of PDLLA-nanofiber composites of rhBMP-2 and rhVEGF_{165} as scaffolds for tissue engineering

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Abstract

Electro spun PDLLA-nanofibers were functionalized with ^{125}I-rhBMP-2 and ^{125}I-rhVEGF_{165}, either by protein-adsorption to, or by incorporation into electrospun PDLLA-nanofibers. Both, adsorbed and incorporated growth factors could be visualized via autoradiography. By fitting the release data according the two phase exponential decay, release rates as well as release-half-lives could be calculated mathematically. Moreover different release rates could be observed, suitable to design multimodal hybrid materials. Loading the PDLLA-nanofibers with rhBMP-2 and/or rhVEGF_{165} would be of benefit in bone regeneration.

1 Introduction

Beginning with 1999 [1] we have immobilized rhBMP-2 by covalent linkage or non-covalent adsorption on metals and ceramic surfaces in amounts of 0.15-8.0 µg/cm² for inducing bone in animals [2-4]. Recently we reported a third method, the incorporation of rhBMP-2 into the poly-(D,L)-lactide (PDLLA) matrix of foamed tablets in the range of 0.8-2.0 mg/g PDLLA in bioactive form [5,6]. In connection with this work we asked the question if a similar incorporation rate of growth factors would be possible into PDLLA-nanofibers, produced by electrospinning [7]. In the work of Schofer et al. [8] with Poly(L-lactic acid) the bioactivity attributable to incorporated BMP-2 remained unclear.

In this paper it will be shown that rhVEGF_{165} can be incorporated into nanofibers by electrospinning in amounts of 1.2 mg/g PDLLA, retaining a high bioactivity. Since in vivo VEGF and BMP-2 are spatio-temporally linked by "angiogenesis-osteo-genesis coupling" via tissue hypoxia and the HIF-1 (hypoxia-inducible factor 1) the incorporation of rhVEGF_{165} into nanofibers was also studied. The interplay of these factors is of central interest in synthesizing bimodal hybrid biomaterials.

2 Materials and Methods

2.1 rhBMP-2 and rhVEGF_{165}

RhBMP-2 prepared in E. coli according to [9] and rhVEGF_{165} according to [10] were obtained from Morphoplant GmbH (Bochum, Germany). Biological activity of rhBMP-2 was determined with MC3T3-E1 cells (alkaline phosphatase activity) according to [11]. Biological activity of rhVEGF_{165} was determined with self-prepared HUVEC cells (dehydrogenase activity) in a proliferation assay (Dojindo Cell Counting Kit-8, Dojindo Molecular Technol. Rockville, MD) [12,13]. The proteins were labeled with ^{125}I by the iodomonochloride method (specific radioactivity: 8000-10000 Counts per minute/µg) as described [13].

For testing the influence of 1,1,1,3,3,3-Hexafluoro-2-isopropanol (HFIP, Sigma-Aldrich, Steinheim Germany) on proteins, rhBMP-2 was the freeze dried and dissolved in a mixture of distilled water (33.3 %) and HFIP (66.6 %) stored therein for 24 h at 7 °C. Subsequently rhBMP-2 was lyophilized and resuspended in sterile PBS-buffer (pH 7.4) for biological activity measurements.

For adsorption measurements PDLLA-nanofiber fleeces were cut into square pieces of (10 mm x 10 mm). Adsorption of ^{125}I-rhBMP-2 and ^{125}I-rhVEGF_{165} from a solution was carried out at pH 4.5 and pH 7.4 respectively (0.1-0.9 mg/ml). Quantification of adsorbed proteins was measured in a gamma counter.

2.2 Electrospinning

The PDLLA-nanofibers were fabricated via electrospinning, using a high voltage power supply with a vertical spinning configuration at room temperature see Asran et. al. [3]. Electrospun fibers were deposited on a fine metal wire netting (100 cm²) consisting of wires (0.5 mm diameter), resulting in a PDLLA-nanofiber-fleece with a distinctive pattern (see fig. 2a). The weight of 1 cm² pieces varied between 1.0-2.5 mg/cm².

Growth factors were incorporated into the nanofiber scaffolds by inclusion in the PDLLA/HFIP solution before electrospinning. Poly-(D,L)-lactide (PDLLA, Resomer® 207 S, Boeringer Ingelheim, Germany) was dissolved (7.5 wt%) in HFIP. RhBMP-2 was dissolved in sucrose/sodium-citrate (14.6 mM sucrose, 5 mM sodium-citrate) buffered solution at pH 4.5. RhVEGF_{165} was dissolved in a PBS (2.7 mM KCl, 1.5 mM KH_{2}PO_{4}, 136 mM NaCl, 8.1 mM Na_{2}HPO_{4}) buffered solution at pH 7.4. The final spinning solution contained a mixture of PDLLA/HFIP (80 %) and aqueous buffered protein solution (20 %), resulting in protein-concentrations of ca. 0.2-0.3 mg/ml. After electrospinning, the amount of incorporated proteins (total PDLLA-fleece = 100 cm²) was quantified in a gamma counter, resulting in ca. 1.22 ± 0.06 mg/g PDLLA (^{125}I-rhVEGF) and 1.69 ± 0.6 mg/g PDLLA (^{125}I-rhBMP-2).
2.3 Protein Adsorption and Release

125I-rhBMP-2- or 125I-rhVEGF165-PDLLA-fleece (~1 cm²) composites were prepared either by protein adsorption or by electrospinning (incorporation). For desorption kinetics a custom made sterile flow-chamber was used [6]. The measurements were performed at 37 °C in a temperature-controlled chamber (CERTOMAT™H, sartorius stedim biotech, Göttingen, Germany). To wash out released protein, the chamber was perfused with sterile PBS-buffer (pH 7.4) at ca. 10 ml/h, maintained by a multiple channel peristaltic pump (205 S, Fa. Watson-Marlow, Willmington, USA). At specified times, the flow chamber containing the 125I-rhBMP-2- or 125I-rhVEGF-PDLLA-fleece composites, was measured in a gamma counter, see Sänger et al. [6]. The data was fitted to a two phase exponential decay function by the PC program Prism 4 for Windows (Fa. GraphPad, San Diego, CA). The calculated half-lives were corrected for the spontaneous decay of 125I (t_{1/2} = 60d).

2.4 Autoradiography

For autoradiography 125I-rhBMP-2 or 125I-rhVEGF was adsorbed/incorporated (as described previously) on PDLLA-fleeces, of ~1 cm² and subsequently fixed on x-ray-film with an adhesive stripon (Amersham Hyper-Film, ECL, Fa. GE-Healthcare, United Kingdom, Buckinghamshire). X-ray-film was exposed for 3 h at -30 °C between two enhancer-foils (Cronex, SR-342, Fa. Dupont, Neu Isenburg). finally the x-ray-film was developed (Fa. Adelfo Chemie, Nürnberg, Germany) and fixed (Fa. Adelfo Chemie, Nürnberg) for 90 s, respectively.

3 Results and Discussion

3.1 Biological activity of rhBMP-2 and rh-VEGF165 in HFIP

As shown in Fig. 1, the half-saturation (K_{0.5}) value of HFIP-treated rhBMP-2 as the parameter for the biological activity [14] is 3.95 ± 0.031 nM and thus practically unchanged in comparison to the native control rhBMP-2, with a K_{0.5}-value of 4.03 ± 0.02 nM. This indicates, that rhBMP-2 is not denatured but moreover that the biological activity of rhBMP-2 is fully retained in HFIP solution and is therefore suitable for electrospinning. VEGF was therefore treated in the same manner.

3.2 Autoradiography images of 125I-rhBMP-2 and 125I-rhVEGF165 PDLLA-nano fleeces

As shown in Fig. 2B-E, adsorbed/incorporated 125I-rhBMP-2 and 125I-rhVEGF165 could be visualized via autoradiography. The blackening of the x-ray-film, caused by the radioactive labelled proteins corresponds to the thickened PDLLA-areas (see Fig. 2A, white marked). However, irregular blackening within the area could be an indication for a varying thickness of the fibers, as previously recognized by weighing square cut fibers of 1 cm².

![Fig. 1: Dose-response measurement of HFIP treated rh-BMP-2 in a MC3T3-E1 cell assay.](image1)

A: native rh-BMP-2 (pos. control)  
B: HFIP-treated rh-BMP-2

![Fig. 2: Visualization of adsorbed/incorporated 125I-rhBMP-2 and 125I-rhVEGF165 on electrospun nanofibers fleeces.](image2)

A: Microscopy image  
B: adsorbed 125I-rhBMP-2  
C: incorp. 125I-rh-BMP2  
D: adsorbed 125I-rhVEGF  
E: incorporated 125I-rhVEGF
3.3 Adsorption of $^{125}$I-rhBMP-2 and $^{125}$I-rhVEGF$_{165}$ on electro-spun PDLLA-fibers

As shown in Table 1 rhBMP-2 and rhVEGF$_{165}$ can be adsorbed in equal amounts of maximally 30 μg/cm$^2$ on PDLLA-nano-fleeces. Based on this amount and a rhBMP-2-adsorption footprint of $\sim$ 9-18 mm$^2$ for both end-on and side-on binding respectively [4] the surface which is covered by the rhBMP-2 molecules corresponds to 148 cm$^2$ (specific surface), resulting in specific adsorption capacity, of 217 ng/cm$^2$ (see [4]).

| Table 1: Concentration dependent adsorption of $^{125}$I-rhBMP-2 and $^{125}$I-rhVEGF$_{165}$ on PDLLA-nanofiber fleeces |
| $^{125}$I-rhBMP-2 | $^{125}$I-rhVEGF$_{165}$ |
| A | B |
| $c_0$ [μg/mL] | $\Gamma_0$ [μg] | $c_0$ [μg/mL] | $\Gamma_0$ [μg] |
| 0.05 | 6.99 ± 1.01 | - | - |
| 0.10 | 7.75 ± 1.97 | - | - |
| 0.15 | 8.27 ± 0.33 | 0.1 | 7.55 ± 0.41 |
| 0.20 | 11.29 ± 2.60 | 0.3 | 16.29 ± 1.48 |
| 0.40 | 16.43 ± 0.81 | 0.6 | 20.76 ± 2.37 |
| 0.60 | 21.58 ± 4.04 | 0.9 | 22.56 ± 3.39 |
| 0.80 | 27.14 ± 3.55 | 1.2 | 30.63 ± 2.27 |
| 0.92 | 32.13 ± 1.54 | 1.5 | 31.89 ± 9.67 |

3.4 Release kinetics of $^{125}$I-rhBMP-2 and $^{125}$I-rhVEGF$_{165}$ from PDLLA-nanofiber fleeces

After preparation of $^{125}$I-rhVEGF$_{165}$ and $^{125}$I-rhBMP-2 PDLLA composites as nanofiber fleeces, it was of major interest to examine the release rate of the proteins.

3.4.1 Release of adsorbed $^{125}$I-rhBMP-2 and $^{125}$I-rhVEGF$_{165}$

The release kinetics were determined, using a continuous-flow method, at 37 °C as described [6]. As shown in Table 2, after a short burst phase adsorbed $^{125}$I-rhVEGF$_{165}$ ($t_{b1/2}$ = 18.7 d) is released at an 11-fold higher rate than $^{125}$I-rhBMP-2 ($t_{b1/2} = 208$ d) in the main phase (s sustained release phase).

| Table 2: Comparison of the calculated kinetic data of the release kinetics* |
| $^{125}$I-rhVEGF$_{165}$ | $^{125}$I-rhBMP-2 |
| Adsorbed | Adsorbed | | | |
| $\Gamma_0$ [μg] | $c_0$ [μg/mL] | $\Gamma_0$ [μg] | $c_0$ [μg/mL] |
| 6.98 ± 0.88 | 0.05 | 7.75 ± 1.97 | 0.10 |
| 8.97 ± 1.53 | 0.15 | 16.29 ± 1.48 | 0.20 |
| 2.31 ± 0.93 | 0.40 | 20.76 ± 2.37 | 0.40 |
| 1.18 ± 0.39 | 0.60 | 22.56 ± 3.39 | 0.60 |
| 0.18 ± 0.03 | 0.80 | 30.63 ± 2.27 | 0.80 |
| 31.89 ± 9.67 | 0.92 | 31.89 ± 9.67 | 0.92 |

*The release of the growth factors can be fitted according the following equation (see Sänger et al. [6]):

$$ y = \text{Span a} \cdot e^{-k_a t} + \text{Span b} \cdot e^{-k_b t} + \text{Plateau (Plateau} = 0). \text{Results are stated as arithmetic mean with standard error (n=3, SEM). ND = not defined. The burst phase is marked as a, main phase as b. Incorpor. Proteins: Burst phase = 1-6%, Main phase 93-99%. Adsorbed Proteins: Burst phase = 7-65%, Main phase 31-92%}. $$

3.5 In vitro biological activity of released incorporated $^{125}$I-rhVEGF

As shown in Fig. 3, incorporated $^{125}$I-rhVEGF$_{165}$ ($K_{0.5}$ = 2.89 ± 0.33 pM) which is released into cell culture medium after 88 d in nanofiber fleeces is still as biologically active as the control rhVEGF (K$_{0.5}$ = 1.82 ± 0.37 pM) from day zero. Since K$_{0.5}$ of rhVEGF$_{165}$ is ca. 1000-fold lower than that of rhBMP-2 (see Fig. 1) rhVEGF activity can therefore still be measured but not the activity of released rhBMP-2.
In vitro testing of the biological activity of adsorbed rh-BMP-2 (5.12 ± 1.29 µg/cm²) showed, that after 4 d in cell culture, a distinctive green fluorescence could be observed compared to the pos. control (native rhBMP-2, 50 mM), see Fig. 4.

4 Conclusions

1. 125I-rhBMP-2 and 125I-rhVEGF165 indicated a two phase exponential release with different rate constants. An 11-fold higher release rate of adsorbed rhVEGF165, versus adsorbed 125I-rhBMP-2, and a ca. 22-fold higher rate, versus incorporated 125I-rhVEGF and 125I-rhBMP-2 may be useful for designing multimodal hybrid biomaterials.

2. Estimated release half-lives of t1/2 ~ 200-400 d may indicate, that protein-release is attributed to the hydrolysis of the PDLLA-fibers.

3. Incorporated rhVEGF165 is biologically highly active after release, indicated by a K0.5-value of 2.89 µM, as parameter for biological activity. Released rhBMP-2 (incorporated) was not amenable to biological activity measurement.

4. Biological activity of adsorbed rhBMP-2 could be shown qualitatively by fluorescence microscopy.

5 References


6 Acknowledgments

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Phagocytic activity of the epithelium in the middle ear – a comparative electron microscopical study in rabbits implanted with various silica-coated Bioverit® II-TORPs for 21 days

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Abstract

Total ossicular replacement prosthesis (TORP) needs to be frequently implanted in a chronically infected middle ear. The continuous release of adequate antibacterial substances by the implant enables the local suppression of bacterial growth without significant side effects. Therefore, the biocompatibility of the locally applied silver as an antibacterial agent has been tested in the middle ear. Dense and nanoporous silica-coated TORPs partly containing silver or covered by a 1% silver sulfadiazine cream were implanted into the middle ear of rabbits. After 21 days the mucosal layer of the middle ear was analysed under the light and transmission electron microscope. Surprising, the epithelium had phagocytosed silica fragments and cream in all experiments. Without any direct contact to the silver-containing implants, apoptotic and necrotic cellular remnants induced the disintegration of the barrier function of the epithelium. The presence of single silver nanoparticles in the mucosal layer suggested a cytotoxic effect of the released silver ions. Further studies are necessary to restrict the damage of the surrounding tissue of the middle ear.

1 Introduction

Previous studies proved the efficiency and biocompatibility of silica coated Bioverit® II used for middle ear prostheses [1,2]. In most cases TORPs have to be implanted in infected environments and therefore require an antimicrobial agent preventing the growth of biofilms and medicating the infectious middle ear [3]. The examination of nanoporous silica loaded with the antibiotic Ciprofloxacin as a cover and drug delivery system revealed positive results in the middle ear of rabbits but comprises the risk of new resistances [3]. Therefore, we wanted to investigate the biocompatibility of silver as an alternative antibacterial agent in the middle ear environment.

2 Methods

Five different groups of TORPs were implanted in 40 New Zealand White male rabbits (Charles River Laboratories, France). This study was permitted by the Lower Saxony State Office for Consumer Protection and Food Safety, Dept. of Animal Welfare, No. 33.9-42502-04-I3/1135 in accordance with the German and European animal welfare legislation. Bioverit® II-TORPs were coated with a nanoporous or dense silica layer, partly containing silver. A further group of nanoporous silica TORPs were covered by 1% silver sulfadiazine cream (Flammazine®, Sinclair Pharma GmbH, Frankfurt am Main, Germany). After 21 days the middle ears were inspected and intact mucosa was explanted. After fixation in 2.5% glutaraldehyde in 0.1 M sodium cacodylate, pH 7.3 the specimens were postfixed with 2% osmium tetroxide, dehydrated with graded ethanol and embedded in epoxy resin. Thin sections stained with 2% uranyl acetate and lead citrate were analysed by the transmission electron microscope Tecnai G2 (Eindhoven, Netherlands). For quantitative analysis the extent of phagocytosed material was estimated in 5 independent sections of the mucosal layer in each animal. The score is defined between 0 (no phagocytosis visible) to 5 (every sectioned cell is filled with at least 10 phagosomes). Mean value and standard deviation of each group were documented and statistically analysed.

3 Results

In macroscopically intact mucosal layer the transmission electron microscope revealed regular phagocytosis of fragments and cream in the epithelial layer of all TORPs containing middle ears. The quantitative analysis demonstrated a significant reduction of the score in the animals with silver-containing implants (Fig. 1).

Figure 1 Quantitative analysis of the extent of the phagocytosis inside the epithelium of the mucosal layer of the middle ear containing TORPs with a different coating for 21 days.
Although a direct contact of the epithelium with the implant’s surface could be excluded, small silver nanoparticles were additionally recognizable inside the cytosol (Fig. 2). Apoptosis and necrosis could be seen more frequently in the TORPS with silver, resulting in a disturbance of the important barrier function of the epithelial layer of the mucosa in the middle ear (Fig. 2).

3 Conclusion

It is well known, that silver ions react as an antibacterial agent by destroying the chromosome, disturbing the oxidative cellular processes and dissolving the bacterial wall [4]. The debate whether metallic silver nanoparticles are also toxic in eukaryotes is still ongoing. The present study demonstrates that the epithelium of the mucosal layer of the middle ear is capable of endocytosis. In all experiments fragments of the coated TORPs were phagocytosed. Compared to the experiments with implants coated with a plain silica layer toxic effects could be predominantly seen in cells with phagocytosed silver-containing material. A study with a freshwater alga clearly demonstrated the cytotoxicity exclusively due to silver ions and no adverse reactions of the metallic silver nanoparticles [5]. Therefore it can be suggested, that the release of silver ions inside the acidic phagolysosomes of the epithelium induced the toxic effects. The intracellularly new formed silver nanoparticles can deliver new silver ions for further adverse effects in the surrounding tissue. Further studies are necessary to optimize the therapeutic profit to bacteria on the implant’s surface without any damage of the lining cell layers.

4 References

Coating of Polyimides for the Improvement of auditory implants

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Introduction
Polyimides represent interesting support materials for the electrodes of auditory brainstem or midbrain implants.¹⁻³ One way to increase the data transmission capacity and with that the performance of the auditory implants is the optimization of the electrode-nerve interface. This can be achieved by reduction of the fibroblast growth onto the electrode support material, whereas the viability of neuronal cells should not be affected. One approach to generate a cell-selective surface modification on polyimides is the photochemical binding of suitable polymers. This work presents the coating of the polyimide Kapton® with various photochemically reactive polymers as well as the investigation of the effect of the polymer coatings on the growth of fibroblasts.

Methods
The photochemically reactive polymers were obtained by polymeranalogous introduction of the photoactive arylazido group into copolymers and the biopolymer chitosan. The polymers were characterized by NMR, IR and UV/Vis spectroscopy. The commercially available polyimide Kapton® was coated with the synthesized polymers via spin or spray coating and subsequent irradiation with UV-light. The characterization of the modified polyimide surfaces was performed using ATR-IR spectroscopy, contact angle measurements, AFM and XPS. The effect of the polymer coatings on the growth of fibroblasts was investigated by cultivation of the murine fibroblast cell line NIH3T3 onto coated and untreated polyimide surfaces, respectively.

Results
The synthesized polymers with the photoactive arylazido groups were thoroughly characterized. The polyimide Kapton® was successfully coated with the synthesized polymers. All polymer coatings cause an inhibition of the growth of fibroblasts as compared to untreated Kapton® (see the following figure). Two polymers even possess anti-adhesive properties.

Conclusion
The polyimide Kapton® was coated photochemically with various polymers. All polymer coatings reduce the fibroblast growth. The effect of the polymer coatings on the viability of neuronal cells is subject of ongoing investigations.

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Simulation of an ossicular chain and middle ear implants with pad

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Introduction

In case of traumatic or pathologic situation it can be necessary to replace the ossicular chain by a middle ear prosthesis. To prevent a possible migration of the implant through the tympanic membrane, cartilage slices are placed between the implant and the tympanic membrane. The aim of this project is to develop middle ear implants with a silicone pad on the eardrum side of the implant, to minimize stresses in the eardrum.

Methods

A human middle-ear which was embedded in epoxy resin was examined using micro computed tomography. On the basis of this data a finite-element model was generated. The eardrum is not visible in the microCT-data. Therefore, it was generated from histological polished sections of the same specimen. The boundary conditions at the eardrum and the stapes footplate were implemented in the model based on the literature. In a second model the ossicular chain was replaced by a middle ear implant with a silicone-pad on the eardrum plate and another one with a cartilage-pad. The material properties of the cartilage were taken from the literature. The material properties of the silicone were measured using tensile tests and simulated using Neo-Hook’s law.

Results

The transmission behaviour of the ossicular chain and the middle ear implants is different, whereas the dynamical behaviour of the implant with a cartilage-pad is similar to that of the implant with a silicone-pad.

Conclusion

The use of a silicone-pad between the headpiece of the middle ear implant and the eardrum is an alternative to the cartilage-pad. The developed method can be used to test different silicone mixtures to get results for the sound transmission which are more like the one of the ossicular chain, than the cartilage pad.

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Experimental study on the biocompatibility of middle ear prostheses with silver nanoparticle-containing silica coatings in rabbits

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In chronic otitis media with destruction of the ossicular chain, prostheses have to be implanted to restore sound transmission. The infected environment promotes biofilm formation, leading to ongoing infection and loss of the implant. Therefore, materials with antibacterial properties are of high interest to protect the implant and to reduce the use of systemic antibiotics.

Silver is known for its antimicrobial activity but there is also evidence for local and systemic toxicity. To examine silver as a possible agent to prevent perioperative infection in middle ear surgery, silver nanoparticles (SNP) were introduced in varying silica coatings and applied on Bioverit II ® middle ear prostheses.

Cytocompatibility was tested in cell culture (mouse fibroblasts) and antimicrobial potential of the coatings was evaluated using fluorescent Pseudomonas aeruginosa.

In order to assess the biocompatibility in the special environment of the middle ear, we implanted 40 male rabbits using prostheses with five different coatings.

One group was implanted with a mesoporous silica film containing SNP , another one with a dense silica film containing SNP, two groups with pure silica films (mesoporous and dense) as negative controls and one group with a mesoporous silica film applied with 1% silver sulfadiazine cream as positive control.

Clinical status was controlled every day. After 21 days, animals were sacrificed to collect the prostheses, middle ear tissue and organ samples for microbiological testing, chemical analyses and light and electron microscopy.

The in vivo testing of the silica + SNP coatings showed good cytocompatibility and antimicrobial effects. During the in vivo study the animals showed good health constitution and no bacterial infection was seen in microbial testing, haemograms and microscopy after euthanasia in any group.

Further in vivo studies on the antimicrobial effectiveness are needed in the attempt to establish SNP as a therapeutic target in middle ear surgery.
Micro- and Nano-patterned Cochlear Implant Electrode Arrays

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Introduction

Micro- and nano-patterns on surfaces are able to reduce the growth of fibroblasts in cell culture. The aim of the current investigation was to transfer these patterns on functional cochlear implant (CI) electrode arrays and investigate these in vivo.

Methods

Micro-patterns were transferred to the electrode arrays during the production process of the electrodes whereas nano-patterns were generated by femtosecond laser treatment of functional electrode arrays. These electrodes were then electrically investigated and implanted in guineas pigs for 4 weeks. Impedance development and hearing thresholds were monitored during this time and at the end of the trial period samples were harvested and prepared for histology.

Results

The reproducibility of the patterns is better when structuring is done after electrode production. Circular micro-grooves in the silicone body of the electrodes result in decreased impedances after 4 weeks of implantation, whereas the reduction in impedance seems to be limited to low frequencies when nano-patterns are investigated in vivo. Hearing thresholds are unchanged compared to implanted controls and none of the tested surface-patterned electrodes caused an increase in insertion forces.

Conclusion

Although the transfer of patterns to functional CI electrodes at a reproducible good quality remains challenging, also the in vivo results show the potential of some of these patterns for a long term impedance reduction.

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Surface Functionalization of Silicone Rubber for Improved Cell Adhesion

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Introduction

The surface properties of biomaterials largely determine the reaction of biological tissue. Silicone rubber (polydimethylsiloxane, PDMS) is widely used for biomedical applications because of its chemical stability and reduced toxicity. However, its surface is characterized by its hydrophobicity and biological inactivity, which corresponds to a low cellular adhesion onto its surface. In order to improve the cellular response of silicone rubber, surface modifications have to be performed.

Methods

In this work, three different activation methods (atmospheric pressure plasma treatment, benzophenone photoinitiation and Caro's acid corrosion) have been performed onto PDMS surface. Every activation was followed by a functionalization with ammonia water, formamide, urea, glycin or hydrogen peroxide. Compositional changes of the surface were analysed by infrared spectroscopy (FTIR) and the wettability was measured with water contact angle (WCA). The cellular response onto the modified PDMS surface was investigated in vitro by the adhesion of cultures of fibroblast cells and by the adhesion of platelets from human blood.

Results

Plasma treatment generated the best hydrophilic properties of the PDMS surface among the three activation methods. The values of WCA have been highly reduced after plasma treatment but activation is not stable due to surface recovery. Functionalization has been proved with FTIR among all activation methods. The surface activation and functionalization induced an increase of the adhesion of platelets but only plasma treated samples could exhibit an improvement of the adhesion of fibroblast cells.

Conclusion

Surface modification of PDMS was successfully conducted and the short-time cellular adhesion was improved especially in the case of the activation with plasma. Blood platelets adhered more than fibroblasts following surface modification. However, further studies have to be performed to investigate the long-term stability of such modifications.
Sputtered micropatterned Nitinol thick films
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Introduction
Today, most NiTi medical devices are manufactured by a combination of conventional metal fabrication steps, e.g. melting, extrusion, cold working, etc., and are subsequently structured by high accuracy laser cutting. This combination has been proven to be very successful; however, there are several limitations to this fabrication route, e.g. in respect to the fabrication of more complex devices, miniaturization, mechanical properties, the combination of different materials or the integration of further functionality.

Methods
The fabrication of micropatterned films by using magnetron sputtering, UV lithography and wet etching has been demonstrated to have a great potential to overcome limitations of conventional device manufacturing.

Results
Nitinol micropatterned thick films can be produced up to 75 µm thickness with high design flexibility, aspect ratio up to 5, smooth edge profile, minimal inclusions in volume fraction and size, outstanding mechanical and fatigue properties. Additionally, this process route allows the production of complex design e.g. 2.5 dimensional or stepwise thickness variations.

Conclusion
The fabrication of micropatterned Nitinol thick films by using magnetron sputtering, UV lithography and wet etching offers a great potential to realize novel applications in the medical, as well as in the non-medical field.
Influence of sodium fluoride on the BSA adsorption on titanium

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Abstract

For a better understanding of the biofilm formation on materials, e.g. in the oral cavity, the influence of sodium fluoride (NaF) on the protein layer formation was investigated by bicinchoninic acid assay (BCA assay) and scanning force spectroscopy (SFS) on titanium. The amount of adsorbed BSA and the adhesion force of BSA (bovine serum albumin) were investigated as a function of different NaF concentrations. In the investigated NaF concentration range the BSA surface concentration showed no significant change, but the measured adhesion forces decreased significantly after addition of 1 ppm NaF in comparison to 0 ppm. Hence, application of NaF might facilitate easier removal of the adsorbed protein layer (biofilm) from the titanium surface during daily oral hygiene.

1 Introduction

In modern medicine biomaterials have become indispensable. They are used in many different kinds of applications, e.g. in reconstructive medicine as dental implants, hip replacements, stents and artificial cardiac valves, but they were also used for contact lenses and many other applications [1]. Because of this widespread use of many different artificial materials like titanium, gold, platinum, steel, glass, silicon, PMMA, PTFE, ceramic and different plastics, it is important to investigate the behavior of these materials as they are brought into the human body. Here not only the corrosion properties, but also the biocompatibility and the behavior of the materials when they are exposed to human body fluids and bacterial suspensions are of great importance. Furthermore some of the medically used metals are also used in biofilm reactors as substrates [2] where they are similarly exposed to a bacteria and protein containing fluid.

The oral cavity is an easily accessible system which can be investigated without invasive methods. It is an open system with constantly changing properties due to food and fluid intake, changes in salivary flow and dental hygiene. If an artificial material is brought into this system a biofilm will be formed on its surface. This biofilm formation can be divided into two phases [3-5]. During the primary phase salivary proteins and glycoproteins adsorb. This is a spontaneous and selective process which occurs within seconds and the initial protein layer is called pellicle [6]. In the pellicle proteins like lysozyme, α-amylase, mucin, carbonanhydrase, lactoferrin and many other salivary proteins as well as plasma proteins like fibrinogen and albumin [7-9] can be detected [4,5]. In the second phase of the dental biofilm formation which proceeds slower than the primary phase other biomolecules adsorb on the primary pellicle layer. In this phase also bacteria adsorb on the pellicle which are responsible for many different dental diseases like caries, periodontitis and periimplantitis [5]. Beside the pathogenic effect that it facilitates bacterial adhesion [4, 5], the pellicle has also beneficial properties. It serves as a lubricant, protects the teeth from mechanical abrasion and acidic attacks. Furthermore it provides Ca²⁺-ions for the remineralization of the enamel surface after mineral loss [4, 5].

In case of the oral cavity titanium is a commonly used implant and restoration material. The dental pellicle layer is daily exposed to NaF in form of toothpaste (typically about 1450 ppm NaF), mouthwash (typically about 500 ppm), fluoridated salt and in some regions also fluoridated drinking water [10]. Also some foods contain significant amounts of fluoride like some sorts of fish and tea [10]. It is known that NaF has a beneficial effect on caries prevention due to the replacement of the hydroxyl-group with fluoride which yields fluorapatite which has a lower solubility. A lower solubility of the enamel surface is associated with a cariostatic effect [11]. Also it has been reported, that the fluoridation of hydroxyapatite results in a change of its electrochemical properties which leads to significantly differences in the adsorption of salivary proteins [12] and so in the initial pellicle layer formation.

In this work the effect of fluoride on the protein adsorption on the commonly used dental restoration and implant material titanium was investigated by BCA assay and SFS using the example of BSA as a model protein. Albumin is an important protein in the oral cavity: it provides protection of the tooth surface against demineralization [13, 14], serves as an important carrier protein and is involved in the regulation of the colloid osmotic pressure.

2 Materials and methods

2.1 Buffer and protein solutions

For all measurements in this work bovine serum albumin (BSA, purity ≥ 98 %, Sigma-Aldrich, Steinheim, Germany) was chosen as a model protein, because of its structural similarity to the human serum albumin (HSA) and its comparatively low costs and good disposability. Furthermore albumin is an important blood plasma protein.
which is present in the salivary pellicle where it contributes to the protective properties of the pellicle layer against erosive demineralization of the enamel [13, 14]. Also the properties of this model protein are well known. It has dimensions of 7 x 4 x 4 nm³ [15]. The isoelectric point of BSA lies between pH 4.7 and pH 4.9 [16, 17]. As a buffer medium in which the protein adsorption experiments were conducted a sodium phosphate buffer with a molarity of 0.1 M was used (components: disodium hydrogen phosphate, purity ≥ 99 % and sodium dihydrogen phosphate, purity ≥ 99 %). For different NaF concentrations NaF was dissolved in buffer until a solution with a concentration of 1500 ppm was reached. All lower concentrations were produced by diluting this solution. All experiments were conducted at a neutral pH value of 7.0. BSA was dissolved in the particular buffer solutions until a protein concentration of 2 g L⁻¹ was reached. All protein and buffer solutions were made with Millipore water (18.2 MΩ cm).

2.2 Substrate material

As a substrate for the protein adsorption experiments dental titanium (constituent parts: 99.30 % Ti, 0.30 % Fe, 0.25 % O₂) (Frios, Mannheim, Germany) was used. The isoelectric point of this titanium was determined to pH 5.05 [18]. The titanium samples for the BCA-measurements had a diameter of 4.70 ± 0.08 mm and a thickness of 0.86 ± 0.04 mm. The titanium samples for the SFS measurements had a diameter of 9.50 ± 0.05 mm and a thickness of 2 ± 0.04 mm. The samples were polished to high gloss with a polishing machine (Phoenix 3000, Buehler, Düsseldorf, Germany) and polishing paper P 4000.

2.3 Bicinchoninic acid assay (BCA assay)

In this work the BCA assay was used to determine the amount of protein which adsorbed on the titanium samples. The BCA assay utilizes a color reaction: the peptide bonds in proteins reduce Cu²⁺ to Cu⁺ whereupon bicinchoninic acid builds a complex with Cu⁺. The here formed complex has a strong optical adsorption at 562 nm wavelength. Due to the fact that the amount of reduced copper is proportional to the number of peptide bonds in the sample, after a calibration measurement the color intensity can directly be correlated with the number of protein molecules in the sample and thus the protein concentration. For the shown experiments the Micro BCA™ Protein Assay Kit (Thermo Scientific, Pierce Protein Research Products, Rockford, USA) was used. A tutorial how the BCA assay is done properly can be found in [19] and a more general recipe for the BCA assay can be found in [20].

2.4 Scanning force spectroscopy

Force spectroscopy measurements on dental titanium samples were performed using a Molecular Force Probe MFP3D instrument (Asylum Research, Santa Barbara, USA).

2.4.1 Scanning force spectroscopy protocol

For the measurements of the adhesion force triangular cantilevers with a spring constant of 0.06 N m⁻¹ were used (Novascan, Ames, Iowa, USA). Instead of a sharp tip a borosilicate sphere with a diameter of 5 µm is mounted on the cantilever. This borosilicate sphere has carboxyl end groups on its surface which allow a covalent attachment of proteins to the surface of the sphere (see section 2.4.1.1). In the shown experiments the adhesion forces for the contact times 0, 1, 2, 5, 10, 20, 40, 60 and 80 s were investigated. For each contact time a total amount of 250 force curves at 5 random positions on the sample surface were measured and averaged. After the measurements the measured adhesion forces were normalized by the radius of the colloidal probes. The shown adhesion forces are mean val-

The titanium samples were incubated in BSA solution with a protein concentration of 2 g L⁻¹ for 1 h at room temperature. The NaF concentration in the protein solution varied here from 0 ppm up to 1500 ppm. After the incubation the samples were rinsed five times in buffer solution with the particular pH value and NaF concentration as the protein solution in which they were incubated. The rinsing step is necessary to remove all loosely bound protein from the surface. It has been shown in another work [21] that five rinsing steps are sufficient to remove all loosely bound protein from the surface so that only the initial protein film remains on the sample surface. After the rinsing process the samples were transferred into Eppendorf tubes with 50 µl RIPA buffer and then incubated for 10 min in the ultrasonic bath. It was shown elsewhere [21] that this procedure removes all proteins from the surface so that the amount of desorbed proteins is equal to the amount of formerly adsorbed proteins. After sonication 100 µl ultrapure water as well as 150 µl BCA-reagent were added to the samples in the Eppendorf tubes. The components were vortexed and then incubated at 55°C for 30 min. After cooling down to room temperature 200 µl of the colored sample solution were transferred into cuvettes. Then the optical adsorption at a wavelength of 562 nm was photometrical measured (Genesys™ 10S UV Vis, Thermo Fisher Scientific, Madison, WI, USA).

To analyze the data the optical extinction values of the calibration curve were plotted against the known protein concentrations and a best-fit line was plotted into the diagram. With help of the slope of the best fit line the protein concentrations of the unknown samples can be determined. The protein surface concentration was declared by scaling the measured protein amount to the size of the sample surface. Each shown measurement point is the arithmetic mean of four measurements and the error bars are the root mean square deviation of these measuring points.
ues and the error bars show the root mean square deviation. The adhesion force was measured for 0 ppm, 1 ppm, 10 ppm and 100 ppm NaF in the buffer solution.

2.4.1.1 Covalent attachment of BSA to colloidal SFM probe

Using the carboxyl end groups on the surface of the colloidal probe (see section 2.4.1) BSA can be covalently coupled to the surface using a zero-length crosslinker. The cantilevers were incubated for 3 h in a mixture of 0.1 M dicyclohexylcarbodiimide (DCC) (Sigma-Aldrich, Schnelldorf, Germany) and 0.1 M N-hydroxysuccinimide (NHS) (Sigma-Aldrich, Schnelldorf, Germany). During this incubation the carboxyl groups of the colloidal probe surface are activated for the crosslinking reaction with the protein molecules. After the incubation the probes were rinsed in DMF to remove all unbound DCC and NHS remains. Afterwards they were rinsed in buffer pH 9.0 to remove all DMF remains. Thereupon the cantilevers were incubated in a 2 g L⁻¹ protein solution at pH 9.0 at room temperature overnight. The crosslinking reaction proceeds without forming any spacer between the reactive groups and the protein. Before the measurements the probes were rinsed again in buffer solution and used immediately.

3 Results

Image 1 shows the results for the protein surface concentration by BCA assay with increasing concentrations of NaF in the buffer solution. The concentration ranges here from 0 ppm NaF up to 1500 ppm NaF.

Image 1 Protein surface concentration of BSA as a function of the NaF concentration determined by BCA

The reference samples which were incubated in protein solution without NaF show a protein surface concentration of 39.1 ng/mm² ± 6.6 ng/mm². The addition of 0.1 ppm NaF to the protein solution has no effect, the protein surface concentration is here 38.3 ng/mm² ± 5.2 ng/mm². Even as more NaF is added there is no significant change in the protein surface concentration of the samples up to a NaF concentration of 100 ppm. Here the protein surface concentration is 39.0 ± 6.4 ng/mm². Until a concentration of 500 ppm NaF in the protein solution there could be a small increase in the surface concentration up to 42.9 ± 9.2 ppm at 500 ppm. From 500 ppm up to 1500 ppm there is again no significant change in the protein surface concentration detectable (1000 ppm: 44.3 ng/mm² ± 4.9 ng/mm², 1500 ppm: 44.2 ng/mm² ± 8.1 ng/mm²).

In conclusion no significant change in the amount of BSA which adsorbs on the titanium sample surface could be observed at low NaF concentrations. At NaF concentrations greater than 500 ppm a small increase in the protein surface concentration could be measured.

The adhesion forces for 0 ppm, 1 ppm, 10 ppm and 100 ppm NaF in the buffer solution measured as a function of the contact time by scanning force spectroscopy are displayed in Image 2.

Image 2 Adhesion forces for 0 ppm, 1 ppm, 10 ppm and 100 ppm NaF in the buffer solution measured by SFS

The measured forces increase with increasing contact time of the colloidal probe with the surface which is a well-known phenomenon [22]. The reason for this increase is the formation of more interaction points between the BSA molecules on the cantilever and the titanium surface. After certain contact times the forces reach a steady state. Now all possible interaction points are established. For different measurements the contact time till the steady state is reached can vary. Here for buffer medium without NaF the steady state is reached after 60 s contact time, just as for 1 ppm and 100 ppm NaF in the buffer solution. For 10 ppm this steady state is already reached after 20 s contact time. For a comparison of the forces at different NaF concentrations the forces at 80 s and thus in the steady state are taken (see Image 3). Without any NaF in the buffer solution the adhesion force is 1.71 10⁻³ N m⁻¹ ± 0.59 10⁻³ N m⁻¹. By the addition of only 1.0 ppm NaF the adhesion forces can be decreased to 0.12 10⁻³ N m⁻¹ ± 0.04 10⁻³ N m⁻¹. If the NaF concentration is further increased the adhesion forces do not decrease any further.
For 10 ppm NaF in the buffer solution the adhesion forces are $0.12 \times 10^{-3}$ N m$^{-1}$ ± $0.05 \times 10^{-3}$ N m$^{-1}$ and for 100 ppm the forces are $0.20 \times 10^{-3}$ N m$^{-1}$ ± $0.10 \times 10^{-3}$ N m$^{-1}$. Already low amounts of NaF seem to decrease the adhesion force between the BSA molecules and the titanium surface dramatically. However a further raise of the NaF concentration over two orders of magnitude do not decrease the adhesion forces any further. Because this appears at very low NaF concentrations which have only very little effect on the ionic strength of the buffer solution this is a real decrease of the adhesion forces and not an effect of a change in ionic strength.

**4 Conclusions**

The amount of BSA adsorbed on dental titanium substrates was investigated by BCA assay as a function of the NaF concentration in the buffer/protein solutions. Furthermore the adhesion force of BSA on dental titanium as a function of the NaF concentration and the contact time was investigated.

For NaF concentrations up to 100 ppm the protein surface concentration of BSA is unaffected by the fluoride ions. At a concentration of 500 ppm a minor increase in the surface concentration could be observed. For the range from 500 ppm up to 1500 ppm, i.e. in the relevant concentration range in dental care products, no change in the protein surface concentration could be seen.

For the scanning force measurements a dramatically decrease of the adhesion force by the addition of 1 ppm NaF can be observed. A further increase of the NaF concentration over two orders of magnitude did not result in a further decrease of the adhesion force.

With regard to preventive dentistry, it is of interest that although the amount of protein on the titanium surface stays constant the adhesion forces of the protein decrease significantly. Thus it is possible that the pellicle layer and the adherent bacteria can be easier removed during daily cleaning processes. In addition to the other tooth-health-promoting effects this is another beneficial effect of NaF applied by toothpastes and mouthrinses.

The same argument would hold for a biofilm reactor with titanium substrates supporting the biofilm. Here an addition of fluoride to the medium would be disadvantageous because the productive biofilm could be destroyed by the shear forces in the flow reactor.

**5 Acknowledgements**

We acknowledge the financial support by the DFG within the projects Zi 487/12, Ha 2718/7-3 and SFB 926.

**6 References**

[19] Pierce Biotechnology, Inc., USA, 8/2002 Ref. 2160412

**Image 3** Tip corrected adhesion forces by SFS in the steady state (80 s contact time)
Material characterization of modified electrospun poly(L-lactide) nanofiber matrices for cardiovascular tissue engineering

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Abstract
Electrospun poly(L-lactide) (PLLA) nanofiber matrices represent potential options for cardiovascular tissue engineering. We performed uniaxial tensile tests, differential scanning calorimetry and scanning electron microscopy in order to investigate the mechanical, thermal and morphological properties of PLLA nanofiber matrices prepared with addition of different concentrations of the non-ionic surfactant Triton X-100. Our results show that this additive leads to substantial changes, e.g. lower crystallization of PLLA for higher surfactant concentrations. No significant differences in material properties were observed after ethylene oxide gas sterilization.

1 Introduction
Clinical treatment of heart valve disease is currently achieved by either surgical or increasingly percutaneous implantation of non-regenerative heart valve prostheses. Application of porcine and bovine pericardium, as used in biological heart valve prostheses has proven to be successful. However, long-term durability and homogeneity of the biological materials are limited. In this light, electrospun polymeric nanofiber matrices may represent an interesting approach for heart valve tissue engineering. Electrospun polymeric nanofiber matrices can be manufactured with high reproducibility and do offer the potential for chemical modification to alter matrix properties. In our present study, an additional surfactant was added to optimize the electrospinning process regarding fiber uniformity and density. PLLA fiber matrices were prepared with different concentrations of the non-ionic surfactant Triton X-100, which has a hydrophilic polyethylene oxide chain (on average with 9.5 ethylene oxide units) and an aromatic hydrocarbon lipophilic group. We determined the influence of surfactant addition on morphological, mechanical and thermal properties of electrospun PLLA nanofiber matrices.

2 Materials and Methods

2.1 Sample preparation
PLLA was supplied as Resomer L210 by Evonik Industries, Germany. We prepared the polymer solution with 0 wt%, 0.1 wt%, 0.5 wt% and 1 wt% of the non-ionic surfactant Triton X-100 (Sigma Aldrich, USA). The PLLA nanofiber scaffolds were manufactured with a needleless electrosprinning process using an NS-LAB 200 (Elmarco, Czech Republic). Sterilization of the samples were carried out with ethylene oxide gas (EtO).

2.2 Characterization methods
2.1.1 Scanning electron microscopy (SEM)
Morphology of the PLLA nanofiber scaffolds was examined with a SEM QUANTA FEG 250 (FEI Company, Germany). The images were taken at various magnifications, and the thickness of the fibers were measured at a magnification of 10,000 using the SEM software.

2.1.2 Uniaxial tensile testing
Uniaxial tensile testing was performed on a Zwicki ZN 2.5 (Zwick, Ulm, Germany). The tests were performed with a 10 N load cell and a crosshead speed of 12 mm/min. During the tensile tests the samples were immersed in physiological saline solution at a temperature of 37°C. We measured the tensile force as a function of elongation. From that the elastic modulus (E) was determined in the linear elastic region via linear regression. Furthermore, the elongation at break (εB) and the ultimate tensile strength (σM) were extracted.

2.1.3 Differential scanning calorimetry (DSC)
Melting behaviour was measured using a DSC 1 Star® system (Mettler Toledo, Greifensee, Switzerland). During the scans, the specimens were exposed to nitrogen gas. The sample weights were in the range of 1.6 – 2.7 mg, and the heating rate was 10 K/min. The data were analyzed with respect to glass transition (Tg), melting temperature (Tm) and degree of crystallinity (χ). For the latter a value of χ100 = 93.7 J/g [1] for totally crystalline PLLA was used.

3 Results

3.1 Influence of EtO sterilization
To evaluate the influence of sterilization on PLLA nanofiber matrices, mechanical and thermal properties were determined before and after an EtO sterilization process. The results for PLLA nanofiber matrices with 0.1 wt% of non-ionic surfactant are shown in Image 1. Red and green bars refer to the unsterile and sterile samples, respectively. All results were averaged over at least n = 5 measurements. For σM, εB and χ there is no substantial difference between sterilized and unsterile PLLA samples. Only for the elastic modulus a comparatively high deviation was observed: 52 ± 9 MPa (sterilized) versus 66 ± 22 MPa (unsterile). However, standard error for this measurement is rather high.
As the sterilization process did not substantially affect the material properties, even for different concentration of the non-ionic surfactant (data not shown), it was excluded from the investigations presented in the following.

3.2 Influence of surfactant addition on nanofiber morphology

Based on SEM, morphological properties of the PLLA nanofibers, namely thickness, uniformity and fiber density, were determined. We focussed the investigation on the influence of surfactant concentration (Image 2).

The untreated nanofibers exhibits a uniform structure with rather constant diameter, see Image 1a. The addition of non-ionic surfactant (Image 1b-d) lead to an increasing density of the nanofibers, as well as to an increase of their thickness. For the latter, a detailed analysis for different surfactant concentrations revealed twice the thickness, e.g. 207 nm for 0.1 wt% (Image 2b), and 404 nm for 1 wt% (Image 2d). Furthermore, merging of individual nanofibers was observed for the highest used concentration of 1 wt% surfactant.

3.3 Influence of surfactant addition on mechanical and thermal properties

Image 3 shows the elastic modulus, ultimate tensile strength, elongation at break and degree of crystallinity in dependence of the surfactant concentration, ranging from 0 - 1 wt%.

The elastic moduli (E) show a substantial increase with increasing surfactant concentration up to 0.5 wt% and from that a constant value of $E = 160 \pm 60$ MPa. Both, tensile strength ($\sigma_M$) and elongation at break ($\varepsilon_B$) increase with higher surfactant concentration, see Image 3b-c. The results ranged from $\sigma_M = 3.22 \pm 0.25$ MPa (0 wt%) to $\sigma_M = 8.4 \pm 1.1$ MPa (1 wt%) and $\varepsilon_B = 50.0 \pm 2.5$ % (0 wt%) to $\varepsilon_B = 84 \pm 8$ % (1 wt%). An opposing trend can be described for the degree of crystallinity. $\chi$ dropped from 57.6 $\pm$ 0.4 % (0 wt%) to 38 $\pm$ 6 % (1 wt%). Further thermal properties, i.e. glass transition and melting temperature, are given in Table 1.
Table 1 Thermal properties of electrospun PLLA nanofiber matrices, manufactured with different concentrations of the surfactant Triton X-100.

<table>
<thead>
<tr>
<th>Surfactant Level</th>
<th>$\chi$ [%]</th>
<th>$T_g$ [°C]</th>
<th>$T_m$ [°C]</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLLA no tenside</td>
<td>57.6 ± 0.4</td>
<td>67.5 ± 0.3</td>
<td>177.5 ± 0.1</td>
</tr>
<tr>
<td>PLLA 0.1wt% tenside</td>
<td>54.5 ± 1.8</td>
<td>53.4 ± 2.2</td>
<td>177.5 ± 2.2</td>
</tr>
<tr>
<td>PLLA 0.5wt% tenside</td>
<td>57 ± 4</td>
<td>47.2 ± 0.4</td>
<td>177.0 ± 2.2</td>
</tr>
<tr>
<td>PLLA 1wt% tenside</td>
<td>38 ± 6</td>
<td>55 ± 5</td>
<td>171.9 ± 1.9</td>
</tr>
</tbody>
</table>

For higher surfactant concentrations the trend for the degree of crystallinity indicate a higher flexibility. The effects in the mechanical behaviour correspond with the morphology of the PLLA nanofiber matrices. Higher additive concentration increases thickness of PLLA nanofiber matrices. Consequently the mechanical properties also increase.

4 Conclusion

Thermal, mechanical and morphological properties of electrospun PLLA nanofiber matrices were assessed with respect to the potential influence of an EtO sterilization process, and the influence of non-ionic surfactant addition during matrix manufacture. Results of EtO sterilized and unsterile nanofibers revealed no substantial differences. Furthermore, different concentrations of the non-ionic surfactant Triton X-100 lead to strong changes regarding morphological, mechanical and thermal properties of the electrospun PLLA nanofiber matrices. In comparison with mechanical properties of established biological tissue materials [2] the results indicate the suitability for application in heart valve engineering. However, biocompatibility remains to be investigated.

Acknowledgement

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References

Operating point analysis of a CHANDLER-loop-system for dynamic \textit{in vitro} hemocompatibility testing

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Abstract

CHANDLER-loop-systems provide a way to generate gravity-driven blood flow for \textit{in vitro}-hemocompatibility testing within partially filled tube rings. There are several limitations for the use of CHANDLER-loop-systems regarding background activation and stationary flow conditions. From those limitations, rollover point for the displaced blood column, resonance frequency of the blood column, blood film transport and resulting blood-air-interface were determined crucial and thus examined. The tube material, loop diameter, tube inner diameter and filling volume were chosen according to previous experiments. Experimental flow analysis at different flow rates and tube diameters were carried out using X-rays for visualization of the phase boundary between blood and entrapped air. Rollover point was detected for a range of parameter combinations (flow rate, tube diameter, loop diameter, temperature/viscosity). An oscillating secondary motion of the blood column inside the device was observed. Resonance frequency and blood transport along the tube walls were calculated and discussed. A set of diagrams for instant experimental design were compiled from the data collected.

The results of this bachelor’s thesis provide guidelines for selecting a suitable operating point for CHANDLER-loop-systems adapted to particular experimental settings and objectives.

Introduction

CHANDLER-loop-systems are widely used in hemocompatibility testing to generate an \textit{in vitro}-blood flow under simulated physiological conditions by means of rotating tube rings, partially filled with whole blood. No additional pumping device is required in this kind of setup, so system related blood damage is minimized. Blood-air-contact during tests exists not only at the menisci but also on the much larger inner surface in the upper part of the tube ring, where a thin blood film is transported along rotational direction during operation. Apart from keeping the blood in an oxygen-saturated, arterial state, this blood film can be disadvantageous, because it reduces the tube’s transparency, thus the displacement of the blood column is no longer visible. At higher rotational speed, required for simulating flow in small arteries, displacement will rise until the aft meniscus reaches the highest point of the loop [Fig. 1]. At this displacement, blood will flow downward on the other side, which leads to undefined flow of blood, with less or no reproducibility. The presented Bachelor’s thesis deals with the visualization of the blood’s displacement inside a CHANDLER-loop apparatus referring to the speed of the rotating loop and thus flow rate. The results serve as guidelines for setting up experiments with stable flow and provide insight into limiting factors in the use of the CHANDLER-loop for experiments aiming at high flow rates.

Methods

In accordance to previous experiments, TygonS50HL tubes with an inner diameter of 2.4 mm and 4 mm are used and a loop diameter of 220 mm is chosen [Fig. 1]. One additional loop diameter (100 mm) is introduced. Loops are filled with citrate-anticoagulated porcine whole blood up to a surface/volume [OV] ratio of 25 cm\textsuperscript{-1} (20 cm\textsuperscript{-1} & 15 cm\textsuperscript{-1}). The housed CHANDLER-loop-system is held at a constant temperature of 36° C. X-radiation is used to visualize the menisci via computed tomography (CT) as well as live-radiography [Fig. 2].

![Fig 1: Definition of the blood column in a loop. The ∆a is defined as high level [°] – low level [°]. D_{i} is the inner tube diameter and d is the diameter of the loop, c is the length of the blood column.](image)

Digital radiographs were obtained using an industrial CT-Scanner (BIR Inc computed tomography), fluoroscopic video data were collected with a C-arm X-ray unit (Philips BV Libra). Rotational speed is increased stepwise until rollover point is reached. The public domain image processor ImageJ was used to quantify predetermined angular dimensions used in further calculations [Fig. 1]. A contrast agent (Barium sulphate) was added to the blood for live-radiography.
The resonant frequency for the blood column was calculated for each filling level according to the formula of a water pendulum.

\[ T = 2\pi \sqrt{\frac{L}{g}} \]  

(1)

Live-radiography was used to quantify the oscillation in displacement of the blood column [Fig. 2].

From the difference in length of the initial blood column and the column of blood in a rotating loop, the portion of blood covering the inner surface of the tube above the menisci was calculated. This inner wall volume was used to define an upper boundary for the optimum range of rotational speed. The inner tube transported wall volume \( \Delta V \) was calculated by the following formula.

\[ \Delta V = \frac{\Delta L \times \Delta \omega}{360} \times \frac{A}{360} \]  

(2)

Results

It is assumed that significant diffusive processes take place up to a layer thickness \( L_c \) of 100 µm, with \( A \) being the surface area covered by the blood film [Fig. 4].

\[ L_c = \frac{\Delta V}{A} \]  

(3)

In this interpretation, only a small fraction of the blood transported on the inner wall of the tube will be exposed to potentially harmful blood/air contact. 10 % is chosen as limit for blood volume transported on the inner wall [Fig. 3]. Depending on the aim of subsequent experiments, this limit may be defined differently.

Several possible boundaries limiting rotational speed, such as first resonant frequency, rollover point and blood film magnitude were calculated or determined experimentally. The results are summarized in diagrams showing the operating range for a CHANDLER-loop-system with each tube diameter. Furthermore, there is entered a characteristic curve, which shows the relation about the number of revolutions and the flow rate [Fig. 5]. The shaded area corresponds to the optimum speed range. The lower boundary represents the physiological flow rate within the particular vessel diameters under pulse at rest. The upper boundary is found in the exemplary transport tube volume, which is 10 % from whole blood volume. Up to a theoretic number of revolutions of 41 min\(^{-1}\) blood/air contact increases.

The tube with 2.4 mm inner diameter exhibits a rollover point at about 38 min\(^{-1}\). The diagram for the tube with 4 mm inner diameter shows an optimum speed range of 10 min\(^{-1}\)–20 min\(^{-1}\). The rollover point is not displayed, because it exceeds the maximum speed of the used CHANDLER-loop-system. The first resonant frequency is at 80 min\(^{-1}\). This frequency is not within the range of the currently anticipated optimum speed range.
Acknowledgement

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Bibliography


Discussion

Despite that flow conditions have substantial impact on background activation and overall results, published descriptions of CHANDLER-loop experiments often lack a plausible explanation for the choice of flow-related parameters. The guidelines and tools provided by this Bachelor’s thesis for designing experiments using a CHANDLER-loop system enable the experimenter to find an optimal combination of the parameters rotational speed, tube diameter, loop diameter and filling volume required for the respective tests. Several possible boundaries that limit the range of stable experiments in the stationary state of flow were identified and theoretically as well as experimentally quantified were collected into diagrams for practical laboratory use. A defined range of parameters of practical significance was evaluated; however any future combination of parameters can easily be obtained following the defined experimental pathway. Complimentary to this work, a study of the damage to the blood as a function of the fluid transport of the tube inner wall should be performed. The results may be used to redefine the optimum speed range for upcoming in vitro hemocompatibility experiments.
Contact Angle Measurement on Dental Implants

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Abstract
Two methods of contact angle measurement are applied on screw type dental implants composed of titanium with different surfaces: The standard Wilhelmy method (dynamic measurement) and an extended sessile drop method (static measurement), where droplets with a volume in the picoliter range are generated. Both methods lead to comparable but in detail different results.

1 Introduction

In the area of dental implantation ultrahydrophilic and micro-structured surfaces based on titanium have demonstrated their benefits in preclinical [1,2] and clinical studies [3]. Despite its importance contact angle measurements on dental implants are still rare, probably because their irregular shape [4]. Commonly small titanium discs or mini plates are used (see ref [5]), however a distinct surface generated on a disk or a plate does not a priori have the same wettability as the same surface generated on a dental implant. So direct contact angle measurements on dental implants would be a substantial benefit. Recently Rupp et al [4] and our group [6] have demonstrated the possibility of measuring contact angles on dental implants by the method of Wilhelmy. Water contact angles are calculated from the forces of immersion and emersion of the sample yielding an advancing and a receding contact angle. By extrapolation of the forces to zero immersion equ. (1) applies.

\[
\cos \theta = \frac{F}{\sigma P}
\]

where \( \theta \) is the contact angle, \( F \) the measured force, \( \sigma \) the surface tension of water and \( P \) the perimeter of the sample. However the perimeter of the implant is not always constant over the immersion depth and not exactly measurable in a simple way, only allowing, at best, the determination of a mean perimeter. Moreover, the Wilhelmy method is an indirect way for contact angle determination because the measured parameter is a force. Thus it would be preferable to measure a contact angle directly on a sessile drop by geometrical methods. However, the standard sessile drop method is not reliably applicable to contact angles < 20° [7]. In addition dosing volumes in the microliter range are not applicable to dental implants in general because the generated drop would be too big for their irregular shape. This method works in a limited way for particular screw type implants with relatively large distances between the threads [4]. This problem can be solved by using a much smaller dosing volume in the picoliter range. Thereby droplets with a diameter in the range of 100 µm are generated which are suitable for a proper analysis of dental implants.

Imaginary Contact Angles

The novel concept of imaginary contact angles was recently developed by Jennissen. Detailed discussions are given in references [8] and [9]. Briefly, imaginary contact angles follow directly from the Wilhelmy equ. (1) when \( \cos \theta > 1 \) because \( \cos (i) = 1.543 \). Resulting contact angles can be interpreted in the following way: The lower the value in real number space the more hydrophilic is the sample and, likewise, the higher the value in imaginary number space the more hydrophilic is the sample. We have suggested the term hyperhydrophilic for rough surfaces displaying extremely high wetting rates [8]. Moreover, baseline corrected contact angles are calculated in order to eliminate the apparent force generated by water impregnation and imbibition. Thus equ. (1) has been extended to equ. (2) [8].

\[
\cos \Theta = \frac{F_W}{\sigma P} + \kappa F_z - F_{imb}
\]

\( \Theta \): contact angle, \( F_W \): Wilhelmy force, \( \sigma \): surface tension of water, \( P \): perimeter of the sample, \( \kappa \): a constant, \( F_z \): the sum of novel unconsidered forces, \( F_{imb} \): apparent force generated by water impregnation and imbibition

Nomenclature
In the following, classical contact angles are denoted by a lower case theta (\( \theta \)). The whole range encompassing both classical and imaginary contact angles is denoted by a capital theta (\( \Theta \)) [6,8].

2 Material and Methods

2.1 Material

Screw type test implants (l = 13 mm, Ø (top) = 5 mm) were manufactured from cp titanium, grade 4, ASTM F67. The shape of the implants is shown in Fig. 1. These machined implants were blasted with corundum (sandblasted surface).

2.2 Surface Enhancement

An ultrahydrophilic and micro-textured surface (the so called SLA surface) was generated by acid etching of
sandblasted implants with 50% H$_2$SO$_4$ at elevated temperature for 1 min in a semiautomatic pilot construction (medSurface dent, RENA GmbH, Gütenbach, Germany) as described previously [6]. The etching process was performed at different flow rates: high flow (SLA-hf), middle flow (SLA-mf) and without flow (SLA-nf). After etching the samples were stored in ultrapure water until contact angle measurement.

2.3 Contact Angle Measurement

The machined and the sandblasted implants were washed with acetone by ultrasonication prior to analysis. In each case n = 4 implants were measured by the Wilhelmy method and n = 4 implants by the Sessile Drop method. In case of the different SLA type surfaces 10 implants of each kind were etched whereas n = 5 were measured by the method of Wilhelmy and n = 5 by the sessile drop method. Before measurement implants, which were stored in water, were immersed in methanol for several seconds and air-dried under a clean bench. The analysis was performed as soon as possible after etching (maximal after 90 min).

2.3.1 Wilhelmy Method

Contact angles by the method of Wilhelmy were measured in ultra pure water on a DCAT 11 EC tensiometer (Dataphysics, Filderstadt, Germany). For contact angle calculation a perimeter of 13.35 mm was used. This is the perimeter of the implant at the point of half immersion (3.5 mm) in accordance with ref [4]. Classical advancing ($\theta_a$) and receding ($\theta_r$) contact angles were calculated on Dataphysic’s SCAT software (Vers. 3.2.2.86). Imaginary contact angles were calculated on a custom-made algorithm in MatLab 7.14 according to [9].

2.3.1 Sessile Drop Method with Picoliter Droplets

Static contact angles ($\theta_s$) were measured by the sessile drop method on an OCA 40 Micro device (Dataphysics, Filderstadt, Germany) containing a Picodrop dispenser head PDH 50, a high speed video system UpOCAHn and a top view system TV-VS. The Picodrop dispenser generates single droplets of water with a volume of around 100 pl. In this investigation for each contact angle measurement 3 single droplets were generated with a frequency of 1000 Hz resulting in water droplets of about 300 pl volume. The diameter of the droplets is in the range between 100 and 200 µm (see Fig 2a). The droplet was monitored by the high speed video camera with a frame rate of 200 Hz and additionally by the top view video system (Fig. 2b). The static contact angle was measured at the point of constant droplet diameter by evaluation of the shape of the drop by ellipse fitting with Dataphysic’s SCA 20 software (Fig. 2a). Contact angles were defined as zero when complete spreading was observed on the top view system and no droplet with constant diameter could be observed sidewide on the high speed camera (Fig. 3). Machined and sandblasted implants were analyzed at 5 locations on each

Fig. 1: Photograph of an implant with SLA surface. It shows the shape of the implant and the locations where sessile drop contact angles were measured.

Fig. 2: Contact angle measurement on a hydrophobic machined implant between two threads. The picodrop was monitored by a high speed camera from the side (left image) and by a snapshot from a video of the top view of the thread with picodrop (right image, see arrow): $\theta_a$ (left) = 79.3°, $\theta_r$ (right) = 80.5°, $\theta_s$ (mean) = 79.9°.

Fig. 3: Behavior of wetting of an implant with a SLA surface between two threads. First line: Monitoring with the high speed camera sidewise showing the surface just before (A) and just after (B) putting the water droplet. In image (B) a water film but no droplet is observable. Second line: Snapshot from the video of the top view system showing the surface before (C) and after (D) putting the water droplet. Complete spreading of the droplet between the two threads is observed. This wetting behavior is defined as $\theta_s = 0°$.

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implant: 3 positions between two threads, on the neck and apical. SLA-type implants were analyzed at 11 locations: 6 positions between two threads, 2 positions on the neck and 3 positions apical (see Fig. 1). Firstly the mean of the right and left contact angle was calculated separately for each measurement. Afterward the mean and standard derivation for each group was evaluated over all location and samples.

3 Results and Discussion

In Fig. 4 representative examples of Wilhelmy profiles of screw type implants with hydrophobic and hydrophilic surfaces are compared. Noticeable are the large hysteresis for the hydrophobic machined implant, the negligible hysteresis for the hydrophilic SLA implant and the large baseline differences in the range of 20 mg for both surfaces. The latter effect can be explained by the fact that large amounts of water are captured between the threads during measurement even in case of the hydrophobic machined surface. For this surface after correction of the additional weight an advancing angle of 83.5° and, interestingly, a high imaginary receding angle of 31.5i° are calculated. In contrast the SLA implant shows hyperhydrophilic properties with high imaginary advancing and the receding angles even after baseline correction.

In Table 1 the Wilhelmy contact angle measurements of the different groups of implants are summarized confirming the results of Fig. 4 for the machined and SLA implants. For the different SLA subgroups no obvious effect of the flow rate on the hydrophilicity is observed. Whereas the classical evaluation with Dataphysic’s SCAT software leads to advancing and receding angles of 0° for all SLA samples, the evaluation by our Matlab algorithm shows a “wettability beyond 0°” with high imaginary contact angles even after baseline correction, thus hyperhydrophilic properties. The sandblasted implants show hydrophobic characteristics similar to the machined implants. However, the baseline difference is considerably smaller for the sandblasted implants (about 13 mg) compared to machined and SLA implants (about 20 mg) but still much larger compared to hyperhydrophilic SLA mini plates (3-4 mg) [9].

In Table 2 the results of the dynamic contact angles obtained by the Wilhelmy method are compared with the static contact angles obtained by the sessile drop method with picoliter droplets. As for the sessile drop method a technique to distinguish between different grades of hydrophilicity for contact angles of 0° is still missing, for the Wilhelmy method only the classical values are shown. In case of the hydrophobic machined and sandblasted implants both methods match well. As theoretically expected a slightly higher advancing contact angle compared to the static contact angle is observed [10]. No obvious differences could be observed between different locations on the implant (on threaded part, on the neck and apical, see Fig. 1). The SLA implants, however, exhibit a more complicated behaviour. Despite of showing hyperhydrophilic properties in respect to the Wilhelmy measurement, by the sessile drop method the mean static contact angle is classical and clearly different from zero. Thereby obviously higher contact angles are observed in apical position compared to the threaded part of the implant. At the latter locations low mean contact angles and high standard deviations are characteristic which does not give a very meaningful impression for the wetting behaviour. A more detailed analysis, as summarized in Table 2, clearly shows that the vast majority of contact angles on the threaded part are zero, but there are also singular hydrophobic locations with contact angles up to 73°. The neck of the implant shows in principle the same behaviour as the threaded part with a considerable tendency to more hydrophobic locations for the neck (not shown in table 2).

The hydrophobic islands on the threaded part and the neck might appear because of contaminations by handling the implants between etching and measurement. Based on the fact that the handling occurs preferable on the neck of the implant, it is logically that this part shows a higher tendency to contamination and thereby hydrophobicity. The higher hydrophobicity at the apical location of the implants, however, is still unclear. No obvious influence of the flow rate can be detected.

Comparing the results by Wilhelmy and sessile drop measurement it seems that the Wilhelmy method only detects the wetting properties on the main area of the threaded part, which is contact water. The properties at apical position and on the neck of the implant have apparently no influence on the Wilhelmy contact angle. It also seems that small hydrophobic islands are not recognized by the Wilhelmy method but only the hyperhydrophilic behaviour of the main part is detectable.
Table 1: Water Contact Angles by the Method of Wilhelmy on Screw Type Implants: Mean ± SD

<table>
<thead>
<tr>
<th>Surface of the implant (n)</th>
<th>Baseline Difference [mg]</th>
<th>Contact Angle by Wilhelmy (Evaluation by SCAT) [°]</th>
<th>Contact Angle by MatLab [°]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>θa (mean ± SD)</td>
<td>θs (mean ± SD)</td>
</tr>
<tr>
<td>Machined (4)</td>
<td>20.8 ± 0.3</td>
<td>77.1 ± 6.8</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>Sandblasted (4)</td>
<td>13.1 ± 0.8</td>
<td>84.9 ± 5.9</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>SLA-hf (5)</td>
<td>21.5 ± 0.5</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>SLA-mf (5)</td>
<td>20.8 ± 1.1</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>SLA-nf (5)</td>
<td>21.8 ± 0.2</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
</tr>
</tbody>
</table>

Table 2: Comparison of Water Contact Angles Obtained by Different Methods on Different Locations on Screw Type Implants with Different Surfaces

<table>
<thead>
<tr>
<th>Surface of the implant</th>
<th>Contact Angle by Wilhelmy (Evaluation by SCAT) [°]</th>
<th>Contact Angle by Sessile Drop (threaded part) [°]</th>
<th>Contact Angle by Sessile Drop (apical) [°]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>θa (mean ± SD)</td>
<td>θs (mean ± SD)</td>
<td>n</td>
</tr>
<tr>
<td>Machined</td>
<td>77.1 ± 6.8</td>
<td>0.0 ± 0.0</td>
<td>4</td>
</tr>
<tr>
<td>Sandblasted</td>
<td>84.9 ± 5.9</td>
<td>0.0 ± 0.0</td>
<td>4</td>
</tr>
<tr>
<td>SLA-hf</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>5</td>
</tr>
<tr>
<td>SLA-mf</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>5</td>
</tr>
<tr>
<td>SLA-nf</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>5</td>
</tr>
</tbody>
</table>

n: number of samples, z (tot): total number of measurements, z (0°): number of contact angles with the value of 0°

4 Conclusion

The standard Wilhelmy method and the sessile drop method with picoliter droplets are both suitable for contact angle measurements on screw type dental implants with complicated irregular shape. For hydrophobic surfaces both method match well. For highly hydrophilic surfaces, however, the sessile drop method gives a more detailed view on the wetting properties. By this method a systematically screening of the surface is possible and the dependency of the contact angle from the location can be detected. Wettabilities beyond zero degree, i.e. imaginary contact angles, are only detectable on rough surfaces by the Wilhelmy method for Young contact angles on the smooth surface of the solid of 0° ~ 0° [8]. The Wilhelmy method is preferable for evaluating the properties of a rough surface as a whole including capillary effects [8].

5 Acknowledgements

The authors gratefully acknowledge Mr. Frank Schienle and Mr. Werner Saule from RENA GmbH for their support concerning the generation of the SLA surface on their platform. Also we thank Mrs. Mariam Madani for contact angle measurements.

6 References

BIOFABRICATION for NIFE – Development of Innovative Strategies for the Prevention and Control of Biofilm-Associated Implant Infections

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Abstract
Despite the great advances in medical research, biofilm-associated infections are still regarded as a serious complication. In implant medicine, bacterial colonization of foreign material surfaces can cause severe inflammation. These infections are thought to be a principle reason for implant function loss and usually require costly surgical interventions that are stressful to the patient. To improve the clinical situation, the BIOFABRICATION project aims to develop and optimize innovative implant surfaces that are specifically functionalized to fight biofilm infections and/or completely abolish microbial adherence.

1 Introduction
The number of inserted medical implants rises each year. This trend is a consequence of the steadily increasing average age of the population in developed countries and the profound advances in medical research. However, these insertion of exogenous biomaterials to substitute for body functions is accompanied by an often higher risk of infection [1]. Contrary to human tissues, these abiotic (implant-) materials are prone to bacterial colonization, as natural defense mechanisms are absent. Implant infections are serious complications that are difficult to treat and stressful to the patient. They are the principle reason for early loss of implant function and implant removal [2]. Bacteria colonize implant surfaces in the form of highly organized microbial communities, known as biofilms. The unique architecture of these biofilms, - especially the embedding of the cells in a matrix of extracellular polymeric substances (EPS) confers high antibiotic resistance [3] - is the reason for that standard therapeutic interventions frequently fail. The BIOFABRICATION project aims at the development of sophisticated, long-lasting and patient-specific implant modifications that prevent or fight surface colonization by bacteria. The objective is to provide long-term protection - in contrast to the short term protection provided by the common approach of functionalizing the implant with antibacterials, which are then gradually released. For this purpose, it is planned to improve the protection of implant devices by combining antibacterial surface structures with stimulus-inducible drug delivery systems.

2 Methods
The target surface structures and the stimulus-inducible drug delivery systems are subjected to extensive antibacterial testing.

2.1 Screening process
All modifications are subjected to pre-screening to distinguish modifications with and without antibacterial activity. This test system is based on the non-fluorescent dye resazurin, that is reduced by bacteria to the fluorescent resofurin (Fig. 1A). Determination of the fluorescence relative to an untreated control allows rapid evaluation of biofilm formation on the target structures.

In a second fine screening step, candidate modifications are analyzed in detail, using mono- and multi-species biofilms in a static test system and/or in flow chambers. For the evaluation of antibacterial effects on complex multi-species biofilms, an oral biofilm model was set up that consisted of the four bacterial species Streptococcus oralis, Veillonella dispar, Porphyromonas gingivalis and Actinomyces naeslundii (Fig. 1B). The in vitro coculture model allows continuous, stable cultivation of biofilms for at least 120h.

For the fine-screening experiments, confocal laser scanning microscopy (CLSM) and live/dead staining is used for the qualitative and quantitative description of biofilm formation. Surface modifications that have successfully passed pre- and fine-screening are subjected to biocompatibility analysis and evaluated in an animal model.

Figure 1 Sketch of resazurin reduction in bacteria (A); SEM image of mature multi-species in vitro biofilm (B)
2.2 Structured surfaces

The generation of bactericidal surface structures is based on already existing surface modifications that are further developed and optimized. The manufacturing process focuses on structures that induce mechanical bactericidal effects [4] or micro structures, e.g. ditch structures (Fig. 2), that efficiently reduce accessible and therefore colonizable areas for bacteria on the substratum.

![Figure 2 SEM image of ditch structured surface (A); CLSM-image of live/dead stained analogous surface (B)](image)

2.3 Drug delivery system

Porous silica nanoparticles (NP) are used as carriers for drug delivery. These are loaded with chlorhexidine digluconate (CHX-Gl) and further functionalized with polymer fibers on the NP surface. The polymer exhibits pH-dependent folding and can either block or open the NP pores [5]. Drug release can thus be controlled and induced in an infection-sensitive manner. The antibacterial effect of drug loaded NP on *Staphylococcus aureus* biofilms has already been proven (Fig. 3).

![Figure 3 Antibacterial effect of 50μg/ml CHX-Gl and CHX-Gl loaded NP on mature biofilms of S. aureus](image)

3 Results

For the BIOFABRICATION project, a complete testing infrastructure was successfully set up to evaluate novel bactericidal implant surface modifications. The first target structures and prototype drug delivery systems have already undergone pre-screening.

4 Conclusion

The first analyses show promising results for the development of new bactericidal implant surfaces. However, before clinical application, these technologies must be optimized by specialists in manufacturing, medicine, chemistry and biology. Close interdisciplinary collaboration is essential.

5 Acknowledgement

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6 References

Biopolymer hydrogels for embedding of living cells and biofabrication of complex tissue equivalents by 3D plotting

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Introduction
Development of 3D tissue equivalents which mimic the complex morphology of living tissues is of high relevance for a variety of applications. The rapid prototyping technology of 3D plotting is applicable for processing pasty biomaterials to 3D scaffolds. This method is characterised by its mild process conditions that allow simultaneous processing of sensitive biological components and therefore opens the possibility to fabricate artificial tissue-like constructs.

Methods
As 3D plotting device the BioScaffold from GeSiM was applied. The hydrogel pastes were prepared by dissolving sterilised biopolymer powders (preferred, a mixture of alginate and methylcellulose) in PBS w/o Ca²⁺ & Mg²⁺. For simultaneous plotting of cells, hMSC were mixed into the paste immediately prior to plotting. After plotting, the scaffolds were stabilised by crosslinking in a CaCl₂ solution and thereafter transferred into cell culture medium for further cultivation. The microstructure was analysed by SEM and viability of the cells by MTT and Live/Dead staining. As a proof of principle, adipogenic differentiation was induced and analysed.

Results
The addition of methylcellulose to low concentrated alginate sols allowed the plotting of real 3D structures with defined geometry and dimensions in the range of centimetres. Crosslinking with Ca²⁺ ions led to stable constructs which can be cultivated over weeks. SEM revealed that the alginate strands exhibit a porous structure caused by dissolution of methylcellulose after gelation of the alginate which might be advantageous for oxygen and nutrient supply. Staining of viable and dead cells 24 h after plotting revealed that most of the cells survived the plotting process. Viable cells have been also detected after cultivation for 21 d indicating the suitability of the hydrogel as cell carrier. Differentiation of the hMSC along the adipogenic lineage has been proven.

Conclusion
The newly developed composite material enabled the fabrication of three dimensional cell-laden matrices with defined geometry and relevant dimensions. By utilising more than one of the three available channels for 3D plotting more complex tissue equivalents can be created, consisting of up to three different cell types, deposited in a spatially highly defined manner.
Evaluation of cell viability and functionality in fresh explanted and incubated porcine arteries for investigation in a perfusion culture system

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Abstract:

Ex vivo arterial perfusion systems are increasingly used in different fields of biomedical research. Previously, we developed a perfusion system to visualize drug distribution in porcine arterial tissue. The present study addresses the viability and functionality of isolated arteries over time, to assure the appropriateness of isolated material for such examinations. Taken into consideration the fact, that the ex vivo experiments on drug release take several hours, we tested the arteries immediately after isolation and following incubation to identify possible degenerative changes. Furthermore, we modified our previous perfusion system, to better mimic the in vivo conditions.

1 Introduction

Atheromatous coronary artery disease (CAD) is a major type of heart disease, characterized by thickening, loss of elasticity and calcification of arterial walls. Being responsible for 50% of all cardiac deaths, it remains the leading cause of mortality worldwide [1]. Over the last two decades, the use of metallic coronary stents has become a standard approach in the treatment of CAD [2]. Unfortunately, the uncoated metallic stents of the first generation, also called BMS (bare metal stent) induce a significant rate of artery re-narrowing. This condition, called restenosis, occurs due to growth of connective tissue in the stent lumen. Drug-eluting stents (DES) have been developed as a strategy to fight cell proliferation and minimize restenosis, thus reducing the requirement for re-intervention [3]. However, there are some adverse effects associated with DES implantation, like delayed healing, long-term endothelial dysfunction and inflammation [4]. Strategies for anti-restenotic drug release and distribution have to be elucidated to enable more selective cell targeting.

In previous studies we developed an in vitro vascular perfusion system to investigate the anti-restenotic drug release and distribution in porcine carotid arteries [5]. In the present study, we examined the functionality of vascular cells in freshly isolated and incubated arteries, according to standard method [6]. Additionally, viability testing was performed to explore the metabolic changes of cells over time. Furthermore, we modified our perfusion setup by optimizing the medium and pressure to achieve effective perfusion, mimicking the in vivo situation.

2 Methods

2.1 Material

Porcine common carotid arteries were obtained from the local slaughterhouse. After removal, arteries were cleaned of fat and connective tissue adherents and dissected into segments. Arteries were then randomly divided into two groups, and transferred for transport into preparation solution or NaCl/Tween20, respectively (Table 1).

2.2 Organ bath setup and testing

Functionality testing was performed either on fresh arteries (immediately after transportation) or following 24 h incubation in preparation solution or NaCl/Tween20 (Table 1) at 4 °C. The carotids were cut helically with regularly spaced passages (Image 1) and strips of 2 cm length and 1 mm width were prepared and mounted in the organ bath containing 25 ml of freshly made Krebs solution (Table 1), which was maintained at 37 °C and gassed with carbogen gas mixture (95% O₂ and 5% CO₂). The strips were fixed by strings at a tissue holder on one end and at a force transducer on the other end.

Image 1 Helical cut of a porcine artery.

Before placing the strips into the organ bath system, the transducer was calibrated to 0 mN and 10 mN using standard masses (Image 2).
After an acclimatization time of 1 h, the organ bath testing started. After adding 3 mL of potassium chloride (KCl) to 25 mL of Krebs solution (corresponding to 150 mmol/L KCl) the alterations in contractile response of the arteries were recorded using a computer program (LabChart, ADInstruments Ltd, New Zealand). After each experiment the exact length and weight of the strip was determined. Using these data and the values of alterations in contractile response after stimulation with KCl, the tension (mN/mm²) for each arterial strip was calculated.

### Table 1 Overview on used solutions and gases.

<table>
<thead>
<tr>
<th>Solution/Gas</th>
<th>Transport/Store solution</th>
<th>Testing solution</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NaCl</td>
<td>NaCl (0.9%/w)</td>
</tr>
<tr>
<td></td>
<td>KCl</td>
<td>TrisOH (0.6%/w)</td>
</tr>
<tr>
<td></td>
<td>Na₂HPO₄ x 1 H₂O</td>
<td>NaH₂PO₄</td>
</tr>
<tr>
<td></td>
<td>NaCl (0.5 M)</td>
<td>MgCl₂ + 6 H₂O</td>
</tr>
<tr>
<td></td>
<td>Hepes (0.5 M)</td>
<td>Glucose</td>
</tr>
</tbody>
</table>

### 2.3 Viability assay

CellQuanti-Blue<sup>TM</sup> assay (BioAssay Systems, Hayward, CA, USA) was used to evaluate cell viability in freshly explanted and in preparation solution or NaCl/Tween20 incubated (up to 96 h) arteries. The vessels were cut into small pieces weighing about 200 mg and incubated with CellQuanti-Blue reagent. After 2 h incubation, supernatant was collected and the fluorescence was measured at 590 nm (excitation wavelength of 544 nm) with the Fluostar Optima plate reader (BMG, Offenbach, Germany). The viability of incubated arteries (%) was calculated relative to that of fresh arteries according to the formula: fluorescent units of incubated tissue x100%/fluorescent units of fresh tissue.

### 3 Results

#### 3.1 Functionality

Using an organ bath system, the vascular reactivity of arterial segments was compared between two conditions, i.e. fresh and 24 h incubated. As shown in Image 3, the arterial tension in response to KCl declined after 24 h incubation, indicating decreased vascular reactivity. The decrease was less pronounced in the group incubated in NaCl/Tween20.

![Image 3 Changes in tension of porcine carotid arteries. Each column represents the mean of 3 experiments ± SEM.](image3.png)

#### 3.2 Viability

Consistently with the functionality test, the CellQuanti-Blue test revealed a decrease in cell viability upon incubation over a period of 96 h. Compared to the fresh arteries, whose viability was set to 100%, the incubated ones exhibited a continuous decrease in viability in preparation solution and NaCl/Tween20 (Image 4). Also here, the NaCl/Tween20 seemed to be more protective to the cells. However, a larger group and statistical analysis are needed to confirm this observation.

![Image 4 Changes in viability of porcine carotid arteries. Each column represents the mean of 3 experiments ± SEM.](image4.png)
4 Conclusion

One of the major focuses of our research group in recent years has been examination of anti-fibrotic and -restenotic drug release and distribution in vitro and in vivo [7]. We have developed a perfusion culture system, allowing for characterization of drug distribution behaviour in isolated porcine arteries [5]. Assuming that these ex vivo experiments are being performed at different time points after tissue isolation, it seemed reasonable to test the viability and functionality of arteries over time. While the organ bath as a functionality test is a widely accepted method to measure contraction and relaxation in isolated arteries, the cell viability assay CellQuanti-Blue is routinely used for monolayer cell culture, and to our knowledge has not been applied to entire artery segments yet. Nevertheless, this test could be successfully established, and used to demonstrate decreasing cell viability with increasing incubation time. It should be mentioned, that the cellular functionality was tested only for one time point (24 h incubation), and further experiments with longer observation time are needed to correlate both test systems properly.

To date, the perfusion system is under permanent validation, in order to achieve optimal conditions, which will more accurately represent the in vivo situation. Recent modification of the system includes a 1.36 m water column to simulate the arterial pressure of approximately 100 mmHg and gassing the external medium with carbogen (Image 5).

To conclude, we established a model and testing system taking into consideration physiological aspects of arteries.

Acknowledgement

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5 References


Image 5 Schematic drawing of the in vitro perfusion culture system with modifications like 1.36 m water column and gassing with carbogen.
Linker-mediated attachment of switchable, drug delivering hydrogels to structured surfaces

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Abstract
The attachment of semiinterpenetrating hydrogels with antibody/antigen crosslinker to implant surface is a way for realizing an implant-associated local drug delivery (LDD) system with stimulus-responsive drug delivery. Based on the works of Miyata et al. we investigated a method of covalently coupling the hydrogel on structured surfaces. The semi-interpenetrating system consisting of polyacrylamide (PAAm) fibers on the one hand and a PAAm network on the other hand was chemically coupled via two different linkers to model implant surfaces. (3-Aminopropyl)triethoxysilane (APTES)/N-hydroxysuccinimide ester of acrylic acid (NSA) and (3-trimethoxysilyl)propyl methacrylate (MPTMS) were tested as linker systems. It could be shown that AA m could be copolymerized to these linker systems as fibers, which are then used as basis for the attachment of the PAAm network. The same principle was successfully used with antibody/antigen coupled semiIPN hydrogels on structured surfaces, provided with isothiocyanate labelled bovine serum albumin (BSA-FITC) filled cavities.

1 Introduction
Contemporary implants are often provided with drug containing coatings which are able to deliver drugs in a controlled and sustained way (1). These coatings can consist of several materials. One method of generating switchable systems is the implementation of proteins or protein-controllable structures in a hydrogel network (2). A variation of protein-coupled hydrogels is the modification of hydrogels with antibody/antigen as crosslinker. It could be shown (3) that this kind of modification results in a switchable hydrogel, whose swelling is dictated via the presence of antibodies or antigens and - in consequence - biomolecule-responsive drug delivery is enabled. For building up an implant-associated LDD system with such properties, the hydrogel needs to be coated on a drug containing implant surface. With this purpose, we investigated the linker-mediated attachment of a thin semiinterpenetrating (semiIPN) polyacrylamide (PAAm) network to model implant surfaces. After testing the deposition of layers of semiIPN PAAm with different thicknesses on glass as model surface, we used evaluated parameters for the attachment of an antibody/antigen-conjugated hydrogel to structured stainless steel samples. These samples were performed with isothiocyanate- (FITC-) labeled bovine serum albumin (BSA) filled cavities, used as model drug for release (Image 1).

2 Methods
2.1 Materials
PAAm hydrogel preparation was performed with AAm (99%), MBA (99%), N,N,N’,N’-tetramethylethylenediamine (TEMED) and ammonium persulfate (APS, 99.99%), all purchased from Sigma-Aldrich, Germany in Dulbecco’s phosphate buffered saline (DPBS, without Ca and Mg, PAA, Austria). As model antibody/antigen coupled in the polymer network, mouse IgG (Jackson immunoresearch, USA) and rabbit anti-mouse IgG (Sigma-Aldrich), were purchased. The modification of antibodies/antigens was conducted with N-hydroxysuccinimide ester of acrylic acid (NSA, Sigma Aldrich). Resulting product of modification was purified with Zeba spin Desalting columns 7k MWCO (Thermo Scientific, USA). Detection of the coupled antibodies/antigens were carried out by fluorescence microscopy with the FITC-labeled secondary antibodies; goat anti-mouse IgG (GAM IgG, Abcam, Cambridge, UK) and goat anti rabbit IgG (GAR IgG, Sigma-Aldrich). Coupling of PAAm hydrogels on glass or stainless steel surfaces was performed with (3-aminopropyl)triethoxysilane (APTES) and NSA on the one hand and (3-trimethoxysilyl)propyl methacrylate (MPTMS) (all purchased from Sigma-Aldrich) on the other hand after having modified surfaces by an oxygen-
plasma treatment. MPTMS coupling takes place in an acetonitrile (ACN, Carl Roth, Karlsruhe, Germany)/acetic acid (J. T. Baker, Central Valley, USA) mixture. As model substrates glass cover slips of 9 mm in diameter and stainless steel substrates of same diameter were used.

2.2 Surface modification

Before preparing the hydrogel, the model surfaces were treated with an oxygen-plasma for the generation of reactive groups on the surface in order to enable covalent attachment of the silane linkers (Image 2a)). The plasma activation process was performed via an oxygen-plasma in a radio frequency (RF) plasma generator (frequency 13.56 MHz, power 50 W, Diener electronic GmbH & Co. KG, Ebhausen, Germany) at a pressure of 0.3 mbar. After plasma treatment, a wet chemical process follows. Either the sample was transferred for 24 h into 4 ml of APTES dissolved in water (0.2 M) (4) (Image 2b) or 24 h into 4 ml of ACN with 0.2 wt% acetic acid and 0.4 wt% MPTMS (5) (Image 2c).

In order to establish a vinyl function, necessary for AAm coupling, onto the APTES modification a second step for modification of the amino group was necessary. Hence, samples were washed in pure water after APTES attachment and brought into a NSA (0.3 mM in DPBS) solution for 1 h (Image 2 b).

Image 2 Surface modification. First step: O₂-plasma based establishment of hydroxyl-groups (a). Second step: Adding of vinyl groups via APTES/NSA (b) or MPTMS (c).

2.3 Hydrogel attachment

For realizing the switchable character of the hydrogel coating, we worked with a semi-IPN hydrogel. The performed two step synthesis is demonstrated in Image 3. The synthesis was carried as follows: 183 µl of a 16.38 % AAm-solution in DPBS was diluted by adding 183 µl DPBS. To this solution 5.25 µl APS (0.1 M in DPBS) and 5.25 µl TEMED (0.8 M in DPBS) were added. After transferring 80 µl to a sample of 9 mm in diameter reaction takes place for 10-30 minutes. After that, 75 µl of this solution were discarded and directly 2-10 µl of a second solution (137 µl of a 48.31 % AAm/MBAA (80/1) in DPBS diluted with 137 µl DPBS and brought to reaction with 17.7 µl APS (0.1 M) and 17.7 µl TEMED (0.8 M)) were added and the complete system was covered with a cover slip.

After 50 minutes the cover slip was removed and the complete system was transferred into DPBS for washing.

Image 3 The two-step attachment of semi-IPN hydrogels to model implant surface. The linkers (green) were copolymerized with AAm (red) aiming at long PAAm fibers (red) on the surface a). In a second step, AAm and MBAA (both blue) were brought to reaction in presence of the grafted fibers for building a network (blue) entangled into the previously attached fibres (b)).

2.4 Bioconjugation

The incorporation of antibodies/antigens into this system was carried out with modified antibodies/antigens. For antibody (antigen) modification of the fibers (network) 100 µl (2 mg/ml antibody (antigen) in DPBS) were brought to reaction with 2.6 µl (6 µl) (0.5 mg/ml (2 mg/ml) NSA in DPBS for 1 h at 37°C. The purified product was transferred into the reaction of PAAm fibers (network), like described before, instead of pure DPBS. The successful incorporation was controlled via fluorescence microscopy (λ<sub>exc</sub>=488 nm, λ<sub>em</sub>=520 nm) with a FluView1000 (Olympus, Hamburg, Germany) after 1.5 h incubation with FITC-labelled secondary antibodies (30 µg/ml in DPBS).

2.5 Thickness measurement

The prepared systems were measured related to the achieved coating thickness with a confocal laser scanning microscope (CLSM) Lext OLS 4300 (Olympus, Hamburg, Germany). In order to increase the afforded difference in refractive indice between model implant surface and hydrogel, glass cover slips were sputtered with gold (SPUTTER COATER, Agar Scientific, Essex, UK) prior to hydrogel deposition.

3 Results and discussion

Performed experiments indicate that the attachment of PAAm semi-IPN hydrogels to implant surfaces is possible and can be sized down to nearby 120 µm in thickness.
Especially three points have been figured out: Firstly, the thickness of the film built up in step 1 lies stable at 0.77 ± 0.08 µm under dry and 0.8 ± 0.1 µm under wet conditions even if reaction time was expanded from 10 to 30 minutes.

Secondly, the volumina of conducted AAm/MBA solutions in step 2 is of immanent importance for thickness of the complete system and correlates in a linear way (Image 4). It could be shown that a volume of 2 µl leads to hydrogel coatings of 121.54 ± 22.3 µm. 5 µl and 9.45 µl lead to unrequested thicknesses nearby 200 µm.

Thirdly the attachment of biomolecules coupled hydrogels onto the surface functions in the same manner like attachment with unmodified hydrogels and could be determined by fluorescence microscopy. These established parameters were used to prepare an antibody/antigen-coupled semiIPN hydrogel on a stainless steel substrate with cavities. This modification works in same manner like on glass substrates and a release of BSA-FITC out of the covered cavities was possible. The coupling of biomolecules into the system could also be determined by fluorescence microscopy (Figure 5).

4 Conclusion

The results indicates that bioconjugated hydrogels could be established in a relative thin manner on structured surfaces. This leads to awareness that gels could be used for generating a system with switchable character of drug release. This principle will now be adapted to thinner coatings and should be tested for the switchable character of release.

5 Acknowledgement

The authors thank Gabriele Karsten for performance of fluorescence microscopy investigations. This work was funded by the Federal Ministry of Education and Research (BMBF) within REMEDIS (FKZ: 03IS2081).

6 References


Image 4 Film thicknesses of hydrogel coatings. The left bar shows thickness of the AAm-fiber coating, while all other bars give an impression of increasing thickness with increasing volume in step 2.

Image 5 Images of a directly incorporated FITC-labelled antibody. 117/1 semiIPN 15% without (a) and with integrated goat pAb to ms IgG (FITC) (b).
Application of electrospun piezoelectric PVDF-scaffolds for nerve regeneration

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Abstract

Piezoelectric polymers have often been studied as films and bulk materials in various medical applications, nevertheless publications of investigations of piezoelectric fiber structures are still limited. In neural tissue engineering some piezoelectric polymers are being investigated as potential scaffolds for supporting the nerve regeneration processes. A promising material is polyvinylidene fluoride (PVDF) because of its proven biocompatibility and piezoelectric properties, which can possibly stimulate the cells ingrowth with its electrical activity when it is mechanically deformed. In a study to prove its suitability to regenerate medical tissue, PVDF was electrospun into fibrous scaffolds by testing different concentrations (20–30 wt.%) of PVDF dissolved in N,N-dimethylformamide (DMF) and acetone (6:4 and 8:2). For the electrospinning process different flow rates (0.3-2 ml/h) and voltages (16–28 kV) were used to produce defined fibrous scaffolds. The structures of the electrospun PVDF scaffolds were observed and analysed with a scanning electron microscope (SEM) and then evaluated for their mechanical properties, biocompatibility and piezoelectric effect. Electrospun PVDF was compared to untreated/raw PVDF pellets with respect to the presence of the nonpolar α-phase and piezoelectric β-phase by using Fourier-transform infrared spectroscopy (FTIR) and differential scanning calorimetry (DSC). The growing of mouse embryonic fibroblasts cells (3T3) was evaluated on electrospun PVDF scaffolds. Electrospun scaffolds with a concentration of 30 wt.% PVDF resulted in the formation of the piezoelectric β-phase with the highest β-phase ratio. 3T3 cells cultured on the scaffolds were well attached as indicated by spread morphology. The results demonstrate the potential for the use of PVDF scaffolds for tissue engineering applications.

1 Introduction

The regenerative medicine represents an alternative to conventional transplantation procedures using polymeric biomaterials with living progenitor cells [1]. Since the 1950s biological and synthetic piezoelectric polymer structures have been investigated (such as poled polyvinylidene fluoride (PVDF), which was discovered by Kawai in 1969 [2]). Our work deals with the preparation and analysis of piezoelectric PVDF electrospun scaffolds and its potential use as a scaffold for peripheral nerve regeneration. Electrospun piezoelectric PVDF scaffolds are designed, manufactured and tested in a series of experiments.

In studies of peripheral nerve regeneration it has been shown that micro electric pulses of films of PVDF positively influence directional axon growth [3][4]. The piezoelectric effect supports and accelerates the axonal regeneration in artificial grafts. The direction of the growth of axons is controlled by the electrical polarization and a faster growing of neurites is induced [3][4].

2 Methods

2.1 Materials

PVDF pellets (M.W. 530 000), N,N- dimethylformamide (DMF) and acetone were purchased from Sigma Aldrich, MO, USA. For the identification of favorable conditions for producing PVDF scaffolds different concentrations of PVDF and various ratios of a mixture of DMF and acetone were prepared in given ratios (Table 1) at 50 ºC and stored at room temperature overnight prior to electrospinning.

<table>
<thead>
<tr>
<th>PVDF (wt%)</th>
<th>DMF (v/v %)</th>
<th>Acetone (v/v %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>15, 20, 25, 30</td>
<td>8</td>
<td>2</td>
</tr>
<tr>
<td>30</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td>30</td>
<td>6</td>
<td>4</td>
</tr>
</tbody>
</table>

Table 1 Solution contents of PVDF in DMF/Acetone for electrospinning process

2.2 Scaffold fabrication

Electrospinning is a suitable method for the preparation of fibrous scaffolds for tissue engineering. It is the fabrication of thin fibers from a polymer solution by using a high voltage electric field [5][6]. The polymer solution is slowly pumped through a nozzle and gets charged into the high voltage electrical field. Due to the positive charge accumulated in the polymer drop, a Taylor cone forms with an emerging polymer jet at the tip. With continuous evaporation of the solvent, the jet solidifies into a polymer fiber that is randomly deposited on a grounded collector. This leads to the elongation of the fiber due to the forces. The stretching process reduces the initial fiber diameter by several orders of magnitude (Image 1).

The stretching process reduces the initial fiber diameter by several orders of magnitude (Image 1).
The final diameter is dependent of the polymer solution and its properties (viscosity, surface tension, conductivity) and the process parameters. Important process parameters are the electrical voltage, the flow rate and the distance between the nozzle and the collector. Also, environmental factors such as humidity and temperature affect the fiber deposition [7][8].

![Image 1 Principle of electrospinning](image.png)

The above mentioned concentrations of PVDF have been tried and tested to get the best properties for the electrospinning process (in terms of the fiber form, stability and the piezoelectric properties). The flow rates, voltages and collector distances were varied.

2.3 Characterization of PVDF scaffolds

2.3.1 Examination of scaffold microstructure

The resulting surface morphology of the scaffolds were observed by using a scanning electron microscope (VP-SEM S-3400 Type II, Hitachi High-Technologies Europe GmbH Krefeld, Germany). The SEM used a resolution of up to 10.0 nm at an accelerating voltage of 15 kV. The micrographs of the scaffolds were captured at various magnifications and then compared. The size distribution of the fiber diameters was determined by the software “image J” (Bethesda, MD, USA).

2.3.2 Mechanical Tensile Tests

Mechanical properties of the electrospun scaffolds were determined by a tensile testing machine (BOSE-Electroforce-LM1-Test-Bench, Bose Corporation, Eden Prairie, Minnesota, USA) using a 10 N-load cell at ambient conditions. Five samples for each scaffold type were prepared in form of a rectangular shape with dimensions of 15 × 10 mm².

2.3.3 Fourier transform infrared spectroscopy

A ParkinElmer FTIR-Spectrum100 Spectrometer (Waltham, Massachusetts, USA) was used to record the infrared spectra of the electrospun PVDF which was compared with untreated/raw PVDF pellets with respect to the presence of the nonpolar α- and piezoelectric β-phase. The crystalline phases present in the samples were characterized in the range between 650 and 1500 cm⁻¹, with 1 cm⁻¹ resolution.

2.3.4 Differential scanning calorimetry

The calorimetric measurements were performed in a DSC (Netzsch DSC 204 F1 Phoenix, Selb, Germany) apparatus. The sample weight of the electrospun PVDF scaffolds resp. raw PVDF pellets was approximately 10 mg. The melting temperature (Tm) and heat of fusion of all samples were determined. The samples underwent a cool-heat temperature cycle program with a cooling and heating rate of 10 K/min from −70 to +250 °C in flushing nitrogen.

2.3.5 Cell attachment measurement

Mouse embryonic fibroblasts cells (3T3) were cultured to confluence in cell culture Dulbecco’s modified Eagle medium. The medium was removed after one day, and the 3T3-cells were seeded on the PVDF electrospun scaffolds (1.5x1.5 cm²), which were sterilized with 100% ethanol, rinsed three times with phosphate buffered saline (PBS) and placed on the bottom of 9-well tissue culture plates. The samples were incubated later with 20% methanethiosulfonate (MTS) in a tissue culture incubator at 37.0 °C with 10% carbon dioxide (CO2). The growing of 3T3-cells was evaluated on electrospun PVDF fiber mats and visualized using fluorescence microscopy (Axiovert 200, Carl Zeiss Imaging Solutions GmbH, Jena, Germany).

3 Results

3.1 Fabrication of PVDF scaffolds

It was possible to produce ultrafine fibers by electrospinning PVDF-solutions. Moreover, concentrations of the solvents DMF and acetone used in preparing the polymer solutions were a main factor to determine the morphology of the electrospun scaffolds. However, DMF has a higher boiling point of 153 °C and stronger polarity that could enable the formation of ultrafine fibers, but its lower volatility could make the process difficult. Therefore using acetone with its higher volatility and lower boiling point of 56.2 °C, could facilitate the generation of the ultrafine PVDF fibers.

3.1.1 Effect of polymer concentration

The concentration of polymer is one of the efficient variables for controlling the ultrafine fiber morphology due to the large variations in the viscosity of the solution caused by increasing the concentration. Image 2 shows SEM micrographs of electrospun scaffolds made from a polymer solution with 30 wt % PVDF in different concentrations of DMF / acetone.

![Image 2 SEM images of electrospun PVDF scaffolds with different DMF / acetone solution ratios. Original magnification 500X. Voltage: 15 kV; concentration: 30 wt .%, flow rate: 1 mL / h; distance nozzle to collector: 25 cm; temperature: 23.1 ° C; humidity: 45%. (a) DMF / acetone = 5:1 (b) DMF / acetone = 8:2, (c) DMF / acetone = 6:4](image.png)
Image 3 shows that by a lower polymer concentration than 20 wt.% a number of beads were visible and no defined fibers were formed. By increasing the concentration from 20 to 30 wt.% the density of bead-fibers decreased and the ultrafine fibers were much more generated.

Image 3 SEM images of electrospun scaffold structure by different polymer concentrations. Original magnification: 500X. Voltage: 15-20 kV, flow rate: 0.5-1.0 ml/h, distance nozzle to collector: 25 cm, temperature: 23 to 25.5 °C; humidity: 47-50%; DMF/acetone = 8:2, (a) 15 wt.%, (b) 20 wt.%, (c) 25 wt.% (d) 30 wt.%

3.2 Mechanical Tensile Tests

The ultrafine fibrous PVDF scaffolds with 100-200 µm in thickness have been tested using the tensile testing system. The tensile direction was along the revolving direction. The stress-strain curves of the all electrospun scaffolds were plotted and the maximum values of tensile strengths and elongations were calculated from the curves (Image 4).

Image 4 Typical tensile stress-strain curves of electrospun PVDF scaffolds prepared from PVDF solution 20, 25, and 30 wt.% respectively in 8.2 (v/v) DMF/acetone

The results revealed that scaffolds produced from PVDF-solution 25 wt.% dissolved in DMF: acetone (6:4) exhibited the highest tensile strength of 138 KPa with 7.5% elongation at break. The lowest tensile value was 56 KPa for the electrospun PVDF 20 wt.% scaffolds and the elongation was 3.7%.

3.3 FTIR spectroscopy

Image 5 shows the FTIR spectrographs of the poled and unpoled PVDF in an adsorption spectra as a function of wavenumbers. Electrospinning of PVDF-scaffolds resulted in a polar β-phase formation, which is relevant for the piezoelectric effect. Scaffolds made from the 30 wt.% PVDF solution in DMF: acetone (8:2) showed the highest β-phase adsorption ratio of 70% at 841 in the FTIR-spectrum.

Image 5 ATR-FTIR spectra of raw PVDF pellet and electrospun PVDF scaffolds 20, 25 and 30 wt.% in DMF:acetone (8:2) to detect the piezoelectric effect (β-phase)

3.4 Differential scanning calorimetry

To examine the crystalline structure and the thermal properties of PVDF in the electrospun scaffolds, DSC was performed to scaffolds and compared with raw PVDF pellets. Image 6 shows the DSC curves of the raw PVDF material and the electrospun PVDF scaffolds. The melting temperature (T_m) and heat of fusion (H) of these samples were determined from DSC curves as shown in Table 2. The crystallinity (X_c) was calculated mathematically [9].

Image 6 Differential Scanning Calorimetry (DSC) spectra of raw PVDF pellet and electrospun PVDF scaffolds with 20, 25 and 30 wt.% in DMF:acetone (8:2) to investigate the piezoelectric effect (β-phase) of the scaffolds

<table>
<thead>
<tr>
<th>PVDF Scaffold</th>
<th>T_m (°C)</th>
<th>H (J/g)</th>
<th>X_c (%)</th>
</tr>
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<tr>
<td>20 wt.%</td>
<td>156.2</td>
<td>73.31</td>
<td>70.02</td>
</tr>
<tr>
<td>25 wt.%</td>
<td>157.0</td>
<td>73.68</td>
<td>70.37</td>
</tr>
<tr>
<td>30 wt.%</td>
<td>157.2</td>
<td>78.37</td>
<td>75.15</td>
</tr>
<tr>
<td>raw PVDF</td>
<td>163.8</td>
<td>64.83</td>
<td>61.92</td>
</tr>
</tbody>
</table>

Table 2 The heat of fusion and peak melting temperature obtained from the DSC curves of electrospun PVDF scaffolds and raw material
The results showed that the electrospun PVDF scaffolds shifted their melting temperatures compared to the raw PVDF. The scaffolds prepared from PVDF solution with 30 wt.% had the lowest \( T_m \). In addition, the thermal enthalpy of the electrospun PVDF scaffolds were higher than the value of the raw PVDF and exhibited a better tendency of crystallization.

### 3.5 In vitro cell study

The morphology of MSCs seeded on the electrospun PVDF scaffolds was evaluated using the above mentioned fluorescence microscopy. The cells were attached and well spread on the PVDF 25 and 30 wt.% scaffolds, and spread in form of spots on the PVDF 20 wt.% scaffolds (Image 7). The PVDF 30 wt.% group had the highest cell number compared to cells on other scaffolds.

![Image 7](attachment:image7.jpg)

**Image 7** Fluorescence microscopy image of mouse embryonic fibroblasts (3T3) on electrospun PVDF scaffolds: a) 20 wt.%, b) 25 wt.%, c) 30 wt.%, and d) Confocal laser scanning microscopy image of PVDF 30 wt.% scaffolds. F-actin is stained green and the nucleus is stained blue.

### 4 Discussion

PVDF is a polymorphic polymer. It has four crystalline modifications and non-crystalline regions, which occurred depending on the processing, thermal and mechanical treatment. It is possible that two or more crystal phases coexist. The different crystal phases are distinguished by the relative positions of the monomers. The \( \alpha \)-phase is formed from the melt during the cooling process, the \( \beta \)-phase is produced by the effect of mechanical stretching of the \( \alpha \)-phase and can be detected in the electrospun PVDF scaffolds. The piezoelectric properties are due to the crystalline arrangement of the \( \beta \)-phase. The \( \alpha \)-phase has no piezoelectric properties. The crystalline phases of PVDF can be analytically distinguished by FTIR and DSC. The characteristic absorption bands for the \( \alpha \)-phase are at 614, 765, 795 and 975 cm\(^{-1}\) [10]. The main absorption bands of the \( \beta \)-phase are at 840, 878 and 1278 cm\(^{-1}\) [10]. This study shows the ability to produce piezoelectric nanofibers of polyvinylidene fluoride with a diameter range of approximately 3 \( \mu \)m. The results of the cell tests showed that the 3T3 fibroblast cells were able to grow on PVDF scaffolds and demonstrated in principle the potential of using PVDF scaffolds for tissue engineering applications. Our next steps will be the investigation of the piezoelectric activity of the scaffolds by developing a test section for directly measuring the piezoelectric properties of the PVDF scaffolds by mechanical stimulation in different load cases. Furthermore in vitro experiments are planned to study neural cells culturing on piezoelectric PVDF-scaffolds.

### 5 Acknowledgement

We gratefully acknowledge the benefit derived from the stimulating discussion with Prof. Dr. med. vet. Kirsten Haaster-Talini from the Hannover Medical School, and the technical assistance of Lutz Dreyer, Shangping Wang, Alexandros Repanas and Lothar Lauterböck from the Institute for Multiphase Processes at the Leibniz University of Hannover, Germany.

### 6 References

Biocompatibility of MgF$_2$-coated MgNd$_2$ alloys in contact with nasal mucosal tissue – in vivo approach

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Introduction

Chronic rhinosinusitis has a prevalence of around 5% (1-19%), depending on the selection criteria. Surgical treatment is often required but, owing to the tubular construction of the paranasal sinus system, is associated with a high restenosis rate and recurring symptoms. The aim of this project is to investigate whether MgF$_2$-coated MgNd$_2$ alloys are suitable materials for paranasal sinus stents.

Methods

The biocompatibility and biodegradation of fluoride-coated magnesium-neodymium specimens, which were attached to the mucous membranes of the porcine frontal sinus (n=12) via median trepanation, were tested over a period of 180 days. In addition to regular endoscopic examination and photographic documentation at an interval of 45 days, histological evaluation of the mucosa and EDX measurements were performed after 45, 90, 135 and 180 days post mortem.

Results

Over the study period, endoscopic examinations revealed a progressive degradation process with extra-epithelial gas formation. After the 135th day, the mucous membrane showed increasing signs of local mucosal hyperplasia and hyperemia, although none of the specimens were completely degraded after 180 days. Histological analysis of the sections, which had been prepared with toluidine blue, indicated neutral osseous metabolism. Following hematoxylin and eosin staining, the microsections exhibited signs of a non-specific foreign-body reaction without any evidence of rejection. EDX measurements detected no magnesium in the mucous membrane.

Conclusion

This study was able to demonstrate that controlled degradation over a period of six months is possible with the magnesium alloy tested. In addition, its excellent biocompatibility forms a good basis for the development of a magnesium stent suitable for treating chronic recurrent sinusitis.

Acknowledgement

We wish to thank the German Research Foundation for its support.
Mesenchymal stem cells for the development of a biohybrid electrode

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Introduction

Missing trophic factors (secreted by hair cells in the healthy ear) lead to the continued degeneration of primary auditory neurons, the spiral ganglion neurons (SGN) even after implantation of a cochlear implant (CI). Moreover, after implantation a sheath of connective tissue (fibrosis) is formed around the CI, impairing the signal transduction between CI and SGN. The development of a biohybrid electrode supporting Mesenchymal Stem Cells (MSCs) in suitable microstructures (preventing their mechanical removal during implantation) could exploit intrinsic immunomodulatory properties of MSCs to promote wound healing after implantation as well as inhibit fibrotic growth around the implant.

Methods

Different square cavities made from thermoplastic polyurethane (TPU) or silicone with or without protein coating were tested as possible growth surfaces for human bone marrow-derived MSCs. Transcriptome analysis was used to analyze changes in gene expression induced in MSCs grown in square structures.

Results

In contrast to silicone structures, square structures of TPU did not negatively influence survival and growth of MSCs. Cultivation of MSCs in these structures alone was sufficient to significantly (> 2-fold as compared to MSCs grown on unstructured TPU) change the expression of more than 500 genes (n = 2). Many of the negatively regulated genes play a role in differentiation processes of MSCs. Among the positively regulated genes a significant number was associated with the cellular response to wounding, for example TGF-β2 and IL1RN. IL1RN is known for its strong anti-inflammatory and anti-fibrotic effects.

Conclusion

Growth in TPU-square cavities seems to inhibit differentiation processes in MSCs and induces an anti-inflammatory and anti-fibrotic phenotype. These results suggest that combining MSCs and cochlear implants in a biohybrid electrode may be a promising advancement in enhancing the functionality of CIs. Future experiments will have to show whether the anti-fibrotic and pro-wound healing effects can be transferred to an in vivo setting.
Biofabrication for NIFE – Personalised implants for the 21st century

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Abstract

BIOFABRICATION for NIFE is a transdisciplinary research consortium, which develops personalised implants based on innovative biomaterials, in order to meet the growing demand for implants and to improve implant safety. The principle goals for clinical use include improvements in biocompatibility and long-term stability, as well as the development of implants which resist infections and prevent chronic immune responses.

1 Introduction

Global demographic development creates a growing need for functionally improved implants. Present implant systems only rarely provide lifelong durability and functionality. Improper ingrowth of implants, rejection of allogenic transplants and clinical infections at the implant-tissue-interface are all frequent causes of premature malfunction [1; 2; 3]. Implant function is often lost. Reoperation may be necessary and this can be life-threatening and be a major financial burden on the health care system. The rising number of implant losses across all clinical disciplines is one of the biggest challenges of modern medicine and emphasises the necessity to develop innovative strategies to optimise implant performance [4; 5; 6].

2 Research infrastructure and methods

In this research initiative, personalised implants are developed in a modular process that starts with basic research and ends with translation into clinical practice (Fig. 1).

In the FABRICATION research modules of BIOFABRICATION, biomaterials of organic and inorganic origin are specifically developed for implant use. The surfaces of implant materials are functionalised using chemical or laser-based methods to achieve antibacterial effects. Alternatively, material surfaces may be modified by these methods to create tissue-specific scaffolds. The final functionalisation is brought about by seeding the implant materials with cells and cultivating the complete construct under controlled conditions in specially designed reactors and containment systems. The whole process possesses a modular character, with the research groups benefiting from constant feed-back and know-how from all other partners.
In the BIO research modules of the initiative, the biocompatibility of all previously developed implant materials is systematically validated. Implant materials are then developed further - either to tissue implants which can be directly transplanted or to solid implants which can resist infection. Finally, systematic studies related to clinical approval are carried out, in order to ensure that the observed scientific results can be translated into clinical use. This modular development strategy is initially being developed for audio-neurological, cardiovascular, dental, musculoskeletal and plastic surgical implants, but will be applied to other organ and implant systems in future projects.

In the development of tissue engineered implants, scaffolds are produced and functionalised to develop patient-specific bioartificial vascular prostheses or implants to replace bone or tooth structures. The modules “Material development” and “Laser-based processes” develop matrices functionalised with growth factors, which allow better ingrowth of the transplanted tissue. All scaffold materials are characterised by cell-biological methods for their biocompatibility (module “Biocompatibility”) and are optimised for their cell-matrix interactions. The long-term biomechanical stability of these scaffolds is established and tested in vitro within a cell-specific stimulation reactor, which is developed by the module “Containment & Control”. Implant stability, healing and perfusion will be analysed by non-invasive optical methods (multi-photon microscopy, sonography, µCT). In the module “Tissue engineering”, the surgical feasibility, sterility and in-vivo integration will be studied in animal models; clinical translation will be evaluated in cooperation with the module “Clinical Translation”.

Solid implants that are resistant to infection are developed via surface structuring by laser-based methods. These methods create defined geometric structures which are non-degradable within the human body and influence the adherence of bacteria and tissue (module “Laser-based methods”). These structures with specific surface architectures can be further functionalised to impart specific implant properties. For example, antibacterial effects or optimal implant healing can be created by using chemical functionalisation, that links...
bioactive molecules to the metallic or ceramic surface or by implementing drug release systems (module “Material development”). The inhibition of implant infections is demonstrated in vitro by microbiological assays and laser-based confocal microscopy (module “Implant-related infections and immunoreactions”). This process is followed by the functionalisation of the complete implant and further in vitro and in vivo testing. Different methods for imaging - such as SLOT and µCT - are applied to document and certify the biological effects of the new implant surfaces on bacteria, cells and living tissue (module “Non-invasive observation”). Biocompatibility, implant stability and advanced conditions for clinical translation will be analysed in cooperation with “Biocompatibility” and “Clinical translation”.

The public acceptance of these fields of biomedical research is studied by the module “PUBLIC”, in order to provide the initiative and other groups working on this field with insights about how the scientific community can translate their work in an accessible manner to the popular media and various public stakeholders.

A program for the interdisciplinary education and training of young scientists has also been established, so as to meet the growing demand for specialists trained in the different fields related to work on implants (module “GRAD”).

3 Conclusion

New research infrastructures have been created within the context of the initiative, which will not only allow active management of the process of implant development up to the stage of clinical translation, but will also make it possible to consider the social acceptance of these developments for the first time - as a significant contribution to the public understanding of science. With post-graduate education and the interdisciplinary training of young scientists, it will be possible to fulfil the growing demand for specialists in research, clinical work and industry.

4 References