Introduction to the Nano World of Cochlear Implant (CI) Surfaces

THESIS

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In the Name of God, the Beneficent, the Merciful

Dedicated to my beloved Mother
for making impossibilities possible for me,
my beloved Dad and
my Love
Abbreviations

AFM       Atomic force microscopy
AiBN      Azobisisobutronitrile
α         Alpha
BP        Bullous pemphigoid
C         Carbon
CaH₂      Calcium hydride
CI        Cochlear implant
Cl⁻       Chloride
cm        Centimeter
CS        Contact stiffness
DMEM      Dulbecco’s modified eagle’s medium
ECM       Extracellular matrix
FA        Focal adhesion
FAK       Focal adhesion kinase
FB        Fibrillar adhesion
FD        Force-Displacement
Fₘₐₓ      Maximum force of detachment
fs        Femtosecond
FX        Focal complex
GFP       Green fluorescent protein
H         Hydrogen
HC        Hair cell
hrs       Hours
IU        International unit
J         Joule
K⁺        Potassium
kHz       Kilohertz
l         Liter
M         Molar
MDBS      Mean distance between two neighbouring local structures
MHz       Megahertz
min.   Minutes
mJ    Millijoul
mm    Millimeter
mN    Millinewton
mol   Mol
MR    Microroughness
mV    Millivolt
mW    Milliwatt
N     Newton
Na⁺   Sodium
NaOH  Sodium hydroxide
Na₂SO₄ Sodium sulfate
nm    Nanometer
NMR   Nuclear magnetic resonance
NR    Nanoroughness
NT    Neurotransmitter
p     Probability
PDGF  Platelet derived growth factor
PDMAA Poly(dimethylacrylamide)
PEtOx Poly(2-ethyl-2-oxazoline)
PI3   Phosphatidylinositol 3
pN    Piconewton
ppm   Parts per million
PRP   Protein repellent polymer
R₄    Average roughness
R₅    Peak to valley roughness
RPTPα Receptor protein-tyrosine phosphatase alpha
r/min Rounds per minute
Si    Silicon
SiO₂  Silicon dioxide
SGN   Spiral ganglion neuron
SNHL  Sensorineural hearing loss
SV    Stria vascularis
TDF-β Transforming growth factor-Beta
<table>
<thead>
<tr>
<th>Symbol</th>
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<tbody>
<tr>
<td>Ti</td>
<td>Titanium</td>
</tr>
<tr>
<td>TiO₂</td>
<td>Titanium dioxide</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet light</td>
</tr>
<tr>
<td>VASP</td>
<td>Vasodilator-stimulated phosphoprotein</td>
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<tr>
<td>WCA</td>
<td>Water contact angle</td>
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<td>µg</td>
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Title: Introduction to the Nano World of Cochlear Implant (CI) Surfaces
Author: Pooyan Aliuos

Abstract
Sensorineuronal hearing loss (SNHL) is caused by the loss of inner ear hair cells and the degeneration of neurites of spiral ganglion neurons (SGN). Despite the degeneration of their neurites, cell bodies of SGNs survive for several years. Cochlear implants (CIs) are used to restore the hearing in patients with SNHL. The function of CIs is based on electrical stimulation of the cell bodies of SGNs via an electrode array, consisting of a number of platinum electrodes, which are enclosed in a silicone electrode carrier. The performance of CIs depends on the state of the electrode-nerve-interface. The encapsulation of CI-electrodes with connective tissues as a result of insertion trauma leads to an increase of the electrode-nerve impedance. Higher power consumption of the implant and a loss of selective stimulation of hearing nerve fibres are caused by the increased impedance. Therefore, the limitation of the adhesion and growth of connective tissue cells on the electrodes and electrode carriers of CIs is desired.

The adhesion of cells to their environment regulates diverse cellular cues, such as migration and proliferation. Cell adhesion is highly dependent on physiochemical properties of material surfaces. These properties can be modulated for selective adhesion and growth of particular cells. The aim of this study was to investigate the effects of physically and chemically modified substrate surfaces on adhesion of fibroblasts as a model for connective tissue cells. In order to investigate the adhesion strength of fibroblasts, single cell force spectroscopy based on atomic force microscopy was used. It was hypothesized that anti-wetting surfaces as a result of altering surface topographies decrease the adhesion of fibroblasts. We further hypothesized that hydrophilic polymer surfaces reduce the strength of cell adhesion.

The results indicate that cells can adjust their morphology in response to material surface topography changes. Consequently, the adhesion of cells correlated with the cell-surface contact area positively. We concluded that physical alteration of surfaces for producing anti-adhesive surfaces should be rather based on reducing the cell-surface contact area than on the reduction of surface wettability. The second hypothesis was proved and the adhesion of fibroblasts to hydrophilic polymer surfaces...
was diminished. It was concluded that due to the leak of proper polymer surface-bound extracellular matrix the attachment of cells to surfaces was inhibited.

In conclusion, the modification of material surfaces influenced the adhesion of cells by two mechanisms: first, by changing the cell-surface contact area and second, by affecting the adsorption of the extracellular matrix components.
Titel: Einführung in der Nano-Welt der Cochlear-Implantat-Oberflächen
Verfasst von Pooyan Aliuos

Zusammenfassung


ist davon auszugehen, dass dies aufgrund einer schwachen Bindung zwischen der extrazellulären Matrix und den hydrophilen Oberflächen geschehen ist.

1. Introduction

1.1. Sensorineural Hearing Loss and the Use of Cochlear Implants

Normal hearing in mammals is based on the mechano-electrical signal transduction through the sensory hair cells (HC) of the inner ear. At their base, inner HCs build synapses with the first auditory neurons, the spiral ganglion neurons. Mechanical sound waves from the environment induce neurotransmitter release at the base of inner hair cells. As a result of neurotransmitter release, receptor potentials are produced and lead post synaptically to the generation of action potentials in afferent spiral ganglion neurons. Sensorineural hearing loss (SNHL) is morphologically characterized by the decline of hair cells, leading to a degeneration of the neuritis of spiral ganglion neurons. Consequently, the generation of receptor and action potentials is interfered, leading to a loss of hearing. SNHL is a profound, permanent and irreversible disorder and might be acquired or congenital. While acquired SNHL can be caused by normal aging process, infections and trauma, ototoxic drugs or noise exposure, congenital SNHL can be set off by German measles, prematurity or jaundice. Despite the degeneration of neuritis of the SGNs, the cell bodies and their projections to the brain stem survive for many years and can be artificially stimulated by synthetic electrical impulses. Therefore, patients with profound SNHL can be provided with cochlear implants (CI).

A CI (Figure 1) is a neuroprosthesis, which uses an array of active electrodes to stimulate cell bodies of spiral ganglion neurons in the cochlea. Therefore, analogue sound signals from the environment are detected by a microphone, which is placed behind the outer ear. The signals are then sent to a speech processor, located outside the skull, which converts the signals to digital codes. By means of electromagnetic induction the processed sound signals are transmitted via a transmitter to a receiver-stimulator, which is placed under the skin near the outer ear and is secured in the bone. Finally, the signals are converted into electrical impulses and are sent to the electrode array via an internal cable to evoke electrical impulses in the cochlea.

The invasive parts of CIs consist of a number (varies in different provided models) of active platinum electrodes and a silicone electrode carrier, which encloses the electrodes. After being implanted into the scala tympani, the active platinum electrodes stimulate the auditory nerve electrically (Figure 1). In response to the evoked electrical impulses, the stimulated neurons generate action potentials and send them to the brain.
Human cochlea is able to detect sound wave frequencies between ~10 Hz and ~16 kHz. The ability to differentiate between various sound frequencies is based on the tonotopic organization of the cochlea. While the base of the cochlea detects high-frequency sound waves and transduces them into electrical signals, the mechanoelectrical transduction of low-frequency sound waves is performed in the apical parts of the cochlea (Figure 2). The electrode array of a CI provides frequency selective hearing by using the tonotopic organization of the cochlea.
1.2. Postoperative Connective Tissue Encapsulation of Implants

The insertion of the electrode array of CIs causes disruptions to the normal anatomical structure in the scala tympani and results in an acute wound. Acute wounds are characterized by a time course of maximally 30 days, in which the wound healing process occurs. The wound healing process is a dynamic and physiologically complex process containing different stages and different molecular and cellular interactions.

Four time dependent stages have been observed for acute wounds: coagulation and haemostasis, inflammation, proliferation and finally wound remodelling. One of the aims of coagulation and haemostasis is to provide the injury site with an extracellular matrix (ECM) for cells, such as macrophages and fibroblasts, which invade the injury site at later stages. The cellular part of wound healing process consists of neurotrophils, macrophages and fibroblasts. While some cells, such as neurotrophils and macrophages are responsible for the phagocytosis to destroy bacteria and foreign bodies, fibroblasts are crucial for the tissue repair and scar formation. Being attracted by different factors such as, transforming growth factor-β (TDF-β) and platelet derived growth factor (PDGF) fibroblasts migrate towards the injury site. By rapid proliferation and producing different ECM factors such as, hyaluronan, fibronectin,
procollagens etc. they further build an ECM, which is rich in its biochemical composition \(^{19,20}\). The newly synthesized ECM is used for further migration and proliferation of cells, crucial for the repair process.

The insertion of the electrode array not only causes a wound in the scala tympani, but also evokes a foreign body response (FBR). Also the FBR is initiated by non-specific protein adsorption from the blood to the implant surfaces. After a fibrin scaffold is formed, macrophages and fibroblasts migrate towards the injury site for phagocytosis and tissue remodelling \(^{21,22}\). The end state of such an acute FBR is characterized by the encapsulation of the implants with connective tissue in order to shield them from the unspecific immune system response of the body \(^{23}\).

As a result of the encapsulation of the CI electrode array with connective tissue, the impedance between the electrodes and the tissue increases \(^{24,25}\). Consequently, the power consumption of the implants increases and a selective stimulation of neurons is negatively affected because the electrical field is scattered probably by the connective tissue or because of higher electrical currents, which are needed to overcome the higher electrode-tissue impedance. Thus, the use of different approaches for reducing the attachment (adhesion) and growth of connective tissue cells on the CI electrodes and electrode carrier is of great clinical interest. In this context, understanding the involved mechanisms in the adhesion of cells to implant surfaces, as the first occurring event after cells come in contact with surfaces, is necessary.

### 1.3. Cell-Substrate Adhesion

The attachment of cells to their environment, termed as cell-substrate adhesion occurs as adherent cells face surrounding surfaces and plays a regulating role in many different cellular processes such as, proliferation, migration, differentiation and gene expression \(^{26}\). For example, the detachment of cells from their substrates induces programmed cell death, apoptosis \(^{27}\).

Cells do not make contacts directly to material surfaces but to ECM components, which adsorb to material surfaces immediately after these come into contact with blood or any other protein-containing media \(^{28,29}\). The ECM, which represents the natural environment of cells, is a complex network consisting of different glycoproteins (e.g. collagens, fibronectins and laminins), proteoglycans (e.g. heparin sulfate), non-matrix proteins (e.g. growth factors) and also a number of matrix-bound glycosaminoglycans, such as hyaluronic acid (hyaluronan) \(^{30,31}\). Cells use the adhesive peptides of ECM
molecules as anchorage points to attach to substrates by using specific adhesion receptors including, integrins, selectins, the immunoglobulin (Ig) super family and other molecules reviewed elsewhere\textsuperscript{32}. Integrins, the best studied adhesion receptors among all cell adhesion receptors, are transmembrane heterodimers consisting of $\alpha$- and $\beta$-subunits, which are associated non-covalently\textsuperscript{26}. Today, 18 $\alpha$- and 8 $\beta$-subunits have been identified to build 24 different heterodimers in mammals\textsuperscript{33}. While the extracellular tails of integrins bind specifically to different ECM glycoproteins, different cytosolic proteins termed as adhesion plaque proteins, link the intracellular tails to the actin cytoskeleton\textsuperscript{34}.

Integrins act as bi-directional transmitters. They transduce physiochemical properties of the ECM into conformational changes at their intracellular tail (outside-in signalling). Due to this conformational change, the intracellular tail evokes binding sites for the adhesion plaque proteins. Integrins also change their affinity for adhesive peptides of ECM molecules in response to mechanical forces, applied by the cytoskeleton (inside-out signalling)\textsuperscript{26,34}. The clustering of integrins occurs immediately after they bind to their ECM ligands and promotes a localized intracellular concentration of signalling molecules, such as non-receptor tyrosine kinases (e.g. focal adhesion kinase (FAK)), which play regulatory role, for example in assembly and disassembly of adhesion sites\textsuperscript{35}. Also members of serine/threonine kinases, for example lipid kinases, such as PI3-kinase and phosphatases (e.g. RPTP$\alpha$) are regulated by integrin binding and clustering\textsuperscript{35}. This signalling pathways lead to the regulation of protein interactions and enzymatic activity by inducing post-translational modifications\textsuperscript{36}, resulting in specified protein recruitment to adhesion complexes and selective linkage of adhesion sites to various downstream signalling cascades, which control the cytoskeletal organization and diverse cellular processes\textsuperscript{35,37}. The communication of cells with the ECM through integrins regulates from one side the cytoskeleton and signal transduction molecules and from the other side leads to an organization of the ECM\textsuperscript{31,35}. Consequently, cells are for example able to migrate over the substrate surfaces. The complex interplay between ECM components, integrins, adhesion plaque proteins and the actin cytoskeleton has been the objective of many studies for understanding the molecular composition, signalling cascades and temporal dynamics of cell-substrate adhesion.

As mentioned before the adhesive machinery of cells consists of different molecules, which interact in order to organize cell response to their environment. In
addition to different transmembrane adhesion receptors, there are more than 100 different adaptor and signalling proteins of the adhesion plaque involved in cell adhesion\textsuperscript{38}. Three functional categories of adhesion plaque proteins have been observed. For example, focal adhesion kinase (FAK) and paxillin have been proposed to be involved in signalling, whereas vinculin and zyxin act as cytoskeletal adaptors. The proteins of the third category play a role in actin regulation (e.g. vasodilator-stimulated phosphoprotein (VASP) and \( \alpha \)-actinin)\textsuperscript{34,39-41}. Beside their molecular complexity, cell-substrate adhesions evoke a highly dynamic and hierarchical assembly and disassembly manner. Zeidel-Bar et al. (2004) divided the temporal dynamics of cell-substrate adhesions in four processes, which vary in their molecular composition, morphology and signalling cues: hyaluronan-mediated adhesion, focal complexes (FX), focal adhesions (FA) and fibrillar adhesions (FB)\textsuperscript{31}.

Hyaluronan is a large linear polymer from the family of glycosamino-glycans and has been shown to mediate the adhesion of cells to their substrates in the very early stages (within seconds) of cell-surface interaction\textsuperscript{31,42,43}. Hyaluronan coats the whole cell membrane externally in the form of a halo with a thickness of 1-5 \( \mu \)m and mediates a soft attachment of cells to substrate surfaces\textsuperscript{31}. The hyaluronan-mediated cell adhesion is a transient process and is replaced by other adhesions in some seconds to a few minutes\textsuperscript{31}.

The next stage of adhesion is mediated by integrin heterodimers and is termed as focal complexes (FX). FXs have been shown to appear under the lamellipodium at the leading edge of migrating cells. They are dot-like adhesion sites with an area size of about 0.25 \( \mu \)m\(^2\). The first appearing molecules in FXs are \( \alpha \)v\( \beta \)3-integrins and phosphotyrosin, followed by talin and paxillin. Vinculin and \( \alpha \)-actinin are observed later in developing FXs together with FAK and VASP\textsuperscript{31}. As the lamellipodium of migrating cells protrude, FXs are formed in great numbers and persist for a few minutes\textsuperscript{44}. As the leading edge advances, new FXs are built in front of the persisting adhesions. Interestingly, as the lamellipodium stops protruding, some of FXs disappear and some of them start growing and transforming into focal adhesions (FA)\textsuperscript{45}.

Focal adhesions are elongated adhesions that are located at the cell periphery and are characterized with a size of 2-5 \( \mu \)m\(^3\). They are not only larger in size than FXs, but also different in molecular composition\textsuperscript{31}. For example zyxin, a regulatory and signalling protein involved in FAs, is absent in FXs\textsuperscript{45}. The transition of FXs to FAs is promoted by the actomyosin-driven contractility, which applies force at cell-matrix
adhesions. This has been concluded after FXs in cells with inhibited myosin light-chain kinase were unable of transition into FAs\textsuperscript{31}. In addition to internal forces, also externally applied forces affect the assembly of FAs. Tan et al. (2010) observed a growth of FAs size in response to externally applied shear forces. Interestingly, the effect was accompanied by a rise of intracellular Ca\textsuperscript{2+} concentration and a rapid recruitment of vinculin to the adhesion sites\textsuperscript{46}. A further interesting finding was the observation of very short assembly and disassembly times for FAs (32.2 ± 2.2 and 60.5 ± 6.0 seconds, respectively), indicating the highly dynamic behaviour of FAs.

By linking the cytoskeleton to the ECM, FAs are able to regulate the physical communication between cells and the ECM. Different mechanisms are involved in the mechanosensory function of FAs. For example, it has been proposed that external forces would unfold the talin binding site for other proteins such as vinculin. This in turn leads to a recruitment of further linking and signalling proteins and also actin monomers to the adhesion site. As a result, FAs are able to sense the physical properties of ECM, such as stiffness and topography\textsuperscript{47}.

The latest stage of cell-substrate adhesion is mediated by fibrillar adhesions (FB)\textsuperscript{31}. FBs arise at the end of FAs, are located in central regions of cells and consist of α5β1 integrins, which bind to fibronectin fibrils\textsuperscript{48}. The concentration of tensin, an actin crosslinking protein, in FBs is higher than in FAs\textsuperscript{34}, indicating the increased development of actin stress fibers. FBs are characterized by a size of 1-10 µm and are anchored at deformable fibronectin, thus they can be displaced by the actomyosin machinery towards the central regions of the cells\textsuperscript{34,49}.

In addition to FXs, FAs and FBs, further cell-substrate adhesion structures, such as podosomes, invadopodia and hemidesmosomes have been observed. Podosomes are small cylindrical structures (~ 0.5 µm diameter), which contain typical focal adhesion proteins, such as paxillin and vinculin\textsuperscript{34}. These structures are mostly found in malignant cells, but also in some normal cells such as macrophages and osteoclasts\textsuperscript{50-52}. They are highly dynamic, consist of integrins and have a life time of 2-10 minutes. Podosomes build clusters, which form rings at the cell periphery\textsuperscript{53}. Invadopodia are similar to podosomes but are more stable\textsuperscript{53}. The function of both podosomes and invadopodia is to contact the substrate surface in order to act as sites for localized proteases secretion and ECM degradation\textsuperscript{53,54}.

Hemidesmosomes are adhesion sites, which connect the extracellular matrix with the keratin cytoskeleton of cells\textsuperscript{55}. In hemidesmosomes, α6β4 integrins are
extracellularly linked to ECM components, such as laminins 5 and collagen IV and intracellularly to the cytoplasmic hemidesmosomal proteins HD1/plectin and BP230, which are in turn connected to the intermediate filaments (keratins 5 and 14). Hemidesmosomes have been shown not only to play important role in maintaining dermo-epidermal adhesion and tissue integrity, but also in intracellular signalling pathways, which modulate cellular processes, such as migration, proliferation and apoptosis.

The dynamic process of cell-substrate adhesion starts with the cell-surface recognition process, which takes less than one second. The distance between the cell membrane and the substrate surface is 1-5 µm at this stage. An early attachment of the pericellular coat of the cells (e.g. hyaluronan) to the substrate surface occurs within seconds. The cell membrane can then approach the surface by reorganizing the pericellilar coat. A contact between the cell membrane and the external surface initiates membrane adhesions, such as focal complexes and later focal adhesions. The transition of early attachment to membrane adhesion takes on the order of some minutes and reduces the cell-surface distance to 10-20 nm, observed in FAs. The last stage is then the spreading of the cell over the surface, which takes some hours and increases the cell-surface area by two orders of magnitude.

1.4. Effects of Physiochemical Surface Properties on Cell Adhesion

Physiochemical surface properties affect the adsorption of ECM components, such as glycoproteins and thus the adhesion of cells. Important parameters for cell-material interactions, such as electrostatic charge of surfaces, their energy, polarity and wettability are determined by chemical composition of material surfaces. For example, the presence of oxygen-containing groups, have been shown to increase the energy, polarity and wettability of material surfaces resulting in improved cell adhesion. The energy of a surface is determined by its polar and non-polar components and can be calculated using contact angle measurements between the surface and a liquid of certain polarity (e.g. water). Polar liquids, such as water are able to spread on polar surfaces, resulting in low contact angles. A low contact angle of water on surfaces indicates hydrophilicity, whereas a high contact angle evokes the hydrophobicity of the surface. Wetting (hydrophobic and hydrophilic) properties of material surfaces affect the adsorption of ECM molecules and thus the cellular adhesion.
It has been shown that not only the presence of adhesive ligands, such as RGD-containing oligopeptides, but also their accessibility for their receptors (e.g. integrins) and their spatial organization over the substrate surface influences the adhesion of cells. On hydrophobic surfaces (anti-wetting surfaces), ECM molecules adsorb in a denatured and rigid form, resulting in inappropriate spatial and geometrical conformation of the molecules for being bound by cell adhesion receptors. Thus, on hydrophobic surfaces cell adhesion and spreading is suggested to be reduced.

The attachment of proteins to highly hydrophilic surfaces has been shown to be performed by relatively weak forces resulting in a decrease of cell adhesion to such surfaces. For example, the adhesion of human mesenchymal stem cells was inhibited on extremely hydrophilic surfaces (oxygen-terminated nanostructured diamond surfaces; contact angle < 2°), whereas cells were able to adhere to less hydrophilic surfaces (native nanodiamond surfaces; contact angle 86°). In another study, poly(dimethylacrylamide) (PDMAA), a polymer with hydrophilic surface properties, was implanted into blood vessels of rabbits and the platelet deposition onto the polymer was investigated at different shunting times. Results showed that PDMAA suppressed the unspecific protein adsorption and platelet deposition at all different shunting times of up to 120 minutes. Nevertheless, other studies showed that the spatial organization of ECM adhesive peptides over surfaces influences the adhesion of cells stronger than the absolute number of adsorbed proteins. For example, Filova et al. (2009) investigated the protein adsorption to substrate surfaces with different wetting properties. Furthermore, adhesion and proliferation of different cell types, such as rat vascular smooth muscle cells and bovine endothelial cells were investigated. Despite the larger number of adsorbed ECM molecules to more hydrophobic acrylic acid surfaces, cells showed better adhesion and larger spreading areas on the more hydrophilic octadiene surfaces. Authors concluded that the ECM molecules were better organized on the more hydrophilic surface, resulting in promoted cell adhesion. It is suggested that on more wettable surfaces ECM molecules adsorb in a more flexible form and can be organized by cells.

While chemical surface properties affect the adhesion of cells by changing the surface energy, polarity and wettability, physical surface properties such as surface topography affect the adhesion of cells by other mechanisms. Surface topography of substrates affects the cytoskeletal organization, migration, proliferation and differentiation of cells. Furthermore, also the expression of adhesion receptors...
(integrins) and different cytosolic FA proteins (e.g. FAK) has been shown to be altered due to topographical changes of substrate surfaces. With respect to the scale of material surface irregularities, surface topographies are divided into four categories: macroroughness (size > 100 µm), microroughness (1 µm < size < 100 µm), submicron roughness (100 nm < size < 1 µm) and nano-roughness (size < 100 nm). Although macro-scaled surface irregularities are beneficial for better mechanical anchorage of implants in the surrounding tissue (e.g. bone), they are too large for cells to be felt.

Microroughness can affect cell behaviour because their dimensions are smaller or in the same size as cells (diameters in suspended state ~ 10-50 µm). Nevertheless, their effects on cells seem to be controversial. While some microstructures have been reported to enhance cell adhesion and proliferation, other studies showed a negative effect of such surface structures on these cellular processes. For example, titanium surfaces with micro-porosities led to increased spreading in rat osteoblasts. Also in another study regarding the growth of human osteoblast-like MG 63 on micro-porous titanium surfaces, cells have been reported to evoke a more differentiated phenotype and generated osteogenetic microenvironment by producing bone growth factors. In the same study the hydroxylation/hydration of titanium surfaces increased the energy as well as the wettability of surfaces, resulting in enhanced cell differentiation. Controversially, Kim et al. (2005) reported incomplete spreading, lower cell numbers and slower proliferation of MG 63 cells on micro-porous titanium alloy (Ti-6Al-4V) surfaces in comparison to flat control surfaces.

The mechanisms of these controversial effects of microroughness are still not understood. One problem in interpretation of the results is believed to be the not well-defined parameters to characterize surface roughness. In nearly all studies, the average roughness (Ra) has been determined for characterizing surface. However, this parameter does not give any information about the shape of surface structures (e.g. pores, grooves etc.), about the tip shape of surface irregularities or about the spacing between different surface irregularities. Hence, for convincing results about the effects of such structures on different cellular processes such as cell adhesion, there is the need of an accurate characterization of sample surfaces, giving information not only about the roughness of the surface, but also about the spatial organization of the structures and the shape of their tips.

Because the size of nanostructures is in the same range as the natural ECM components, these surface topographies promise extensive effects on cellular processes.
such as adhesion and proliferation\textsuperscript{61}. Therefore, the effects of nanostructures on cellular behaviour have been widely investigated using many different cell types. For example, it has been shown that wetting properties of nanostructures affect the response of primary rat calvarial osteoblasts\textsuperscript{81}. Cells were cultured on two different titanium nanostructures of the same size (~ 10 nm) but different wetting properties (anatase TiO\textsubscript{2}; WCA ~ 60° and amorphous TiO\textsubscript{2}; WCA ~ 90°). The adhesion, spreading and proliferation of cells were enhanced on more wettable anatase surfaces\textsuperscript{81}. In another study, a relatively hydrophobic terpolymer (WCA ~ 100°) was shown to be inert against cell adhesion and growth. By mixing the terpolymer with carbon nanotubes, surface nanostructures were generated. The generated nanostructures promoted the adhesion and the spreading of MG63 osteoblasts\textsuperscript{82}.

A further phenomenon, observed on nanostructures was the regulation of cell response to the nanostructures by the dimensions of the structures and their spatial spacing over the surface\textsuperscript{64,83-87}. For example, while nanoislands with a height of < 20 nm promoted the adhesion of endothelial cells\textsuperscript{84}, fibroblasts\textsuperscript{85} and mesenchymal stem cells\textsuperscript{87}, same structures with a height of ~ 50 nm were anti-adhesive for fibroblasts\textsuperscript{83}. In another study, performed by Arnold et al. (2004), an adhesive peptide of ECM was mobilized on nanostructures with a diameter of ~ 8 nm, which were small enough to perform a binding site for only one integrin heterodimer. It was shown, that a distance of more than 73 nm between two binding sites led to an inhibition of integrin clustering, resulting in diminished adhesion and spreading of MC-3T3 osteoblasts and also murine 3T3 fibroblasts\textsuperscript{64}. These findings were proved later by Park et al. (2009), where an spacing of 50-100 nm between binding sites resulted in reduced adhesion of different stem cell types. Interestingly, a spacing of 15 nm lead to enhanced adhesion and proliferation of all investigated cell types\textsuperscript{88}. Thus, the spacing of ~ 70 nm between two different integrin-ligand connections could be considered as a universal surface geometry, which probably leads to increased adhesion and spreading of different cell types.

As described in this section, the adhesion of cells to physically and chemically altered surfaces is affected by different mechanism. Nevertheless, surfaces with highly hydrophilic or extensively hydrophobic properties seem to hinder the adhesion of cells in the same way, by affecting the adsorption of ECM components. Since, both physical and chemical surface properties affect the adhesion of cells, surfaces with combined effects could lead to special favourable effects. As an example, Ranella et al. (2010)
showed that the adhesion and spreading of NIH3T3 fibroblasts on laser irradiated silicone microstructures correlated positively with surface energy and wettability.

Many different qualitative and quantitative methods have been developed and used to investigate the adhesion of cells to substrate surfaces. Immunocytochemistry is the most used qualitative method to characterize cell-substrate adhesion. For example, immunocytochemistry and western blotting have been used to investigate the molecular complexity of cell adhesion. Furthermore, the dynamic assembly and disassembly of focal adhesions has been investigated by combining immunofluorescence and video microscopy. Also new insights into the vertical organization of the adhesion plaque (containing talin, vinculin, FAK etc.) was recently shown by using super-resolution light microscopy to observe fluorescently labelled molecules with an accuracy of less than 10 nm. Nevertheless, the outcome of such experiments gives no information about mechanics of cell adhesion, such as the strength of different binding sites between integrins and their ECM ligands or the amount of the mechanical force that is needed to separate cells from their substrates.

Processes of the cell membrane have been considered to be dependent on molecular forces between different associating molecules. Hydrophobic/hydrophilic, electrostatic, van der Waals and hydrogen bonding interactions evoke the typical interacting forces between cell membrane (or any biological surface) and external surfaces. Thus, to understand the language, which describes how cell surfaces are bound to external surfaces, it is of great interest to measure the occurring forces at the cell-substrate interface. To measure adhesion forces, different methods have been developed, all with their advantages and disadvantages. Among all developed methods, single cell force spectroscopy (SCFS), which is based on atomic force microscopy (AFM), is the only one that facilitates the detection of mechanical forces in a proper range for measuring adhesion forces of entire cells and also the strength of single cell-substrate bindings, such as receptor-ligand bonds.

1.5. Single Cell Force Spectroscopy (SCFS)

Since its invention in 1986, the atomic force microscope (AFM) has been used in many application fields, such as chemistry, physics and biology. Using a thin lever (cantilever) of certain stiffness as a mechanical sensor, piezo-electric actuators for moving the cantilever with nanometric accuracy and electronic controllers for precise feedback, the AFM is able to sense substrate surfaces of various stiffness at very small
dimensions (a few nanometers (nm) of displacement and a few piconewtons (pN) of forces).

The function of AFM is based on detecting the vertical and lateral deflections of the cantilever from its position by means of a laser beam and a segmented photodiode. For this, a laser beam, which is focused on the back side of the cantilever, is reflected back to a segmented photodiode. The alignment of the laser beam in the middle of the segmented photodiode causes an electrical potential in the photodiodes. After the cantilever is moved towards the surface and is pressed onto this by means of piezo-electric actuators, a defined cantilever deflection resulting in an electrical potential change in the photodiode (setpoint) is reached. The change of electrical potential is induced by the deflections of the laser beam from the middle of the segmented photodiode. As the cantilever with its sharp tip is moved by the piezo-electric actuators on the x-y plane over the substrate surface, the tip is moved up- and downwards by the surface irregularities, resulting in cantilever deflection from its position and consequently to changes of electrical potential in the photodiodes. The piezo-electric actuators are then regulated in such a way to keep the setpoint constant during the whole scan process. Thus, the movement of the piezo-electric actuators to compensate the cantilever deflections gives indirect information about the surface topography.

Using the fact that the sensor of an AFM is a very soft and thin lever, the device can also be used to measure forces with an accuracy of a few piconewtons (pN). By knowing its spring constant (SC), the deflections of the cantilever can be converted into forces using Hooke’s law, which states that the force applied to a lever is equal to lever displacement multiplied with its spring constant (SC). The SC of cantilevers can be determined by different methods, such as the method of thermal noise analysis. In this method the behaviour of the cantilever, to oscillate at its resonance frequency at a defined temperature is used to measure the thermal noise. This behaviour of cantilevers originates from the oscillations of their atoms in the silicon crystal (cantilevers are normally made of silicon). Because the deflection of the cantilever and the thermal noise of the medium are known, the equilibrium between oscillations of cantilever and those of the medium can be used to measure the spring constant of the cantilever. In single cell force spectroscopy (SCFS), very soft and tipless cantilevers (SC ~ 0.05 N/m) are used to sense forces in the range of 5 \times 10^6 \text{ pN}, suitable for measuring adhesion forces of entire cells and also for detecting the rupturing forces of single receptor-ligand bonds (< 80 pN).
In this method, after a single cell is attached to a functionalized cantilever and is moved towards the substrate surface (extension) until the surface is reached by the cell periphery. At this position the cell is then kept in contact with the surface due to a defined force (setpoint), applied onto the cell by the cantilever. After the cell interacted with the surface for a certain time (interaction time), it is detached from the substrate surface by the cantilever as it is moved away from the surface by means of piezo-electric actuators (retraction). Using the vertical deflection of the cantilever (evokes the applied force) as a function of the position of the piezo-electric actuators, the experiment is illustrated as a force-displacement (FD) curve (Figure 7).

Using FD-curves different parameters can be observed and studied. For example, the peak of the retraction curve represents the maximum force of detachment ($F_{\text{max}}$) of the examined cell. This parameter is considered as the most convincing one to characterize the overall adhesion of single cells\textsuperscript{103}. The jumps in the retraction curve, representing mechanical tensions, occurring as single binding sites between cells and substrate surfaces are ruptured, can be divided into at least two different sections: a first section occurring normally at the very beginning of the retraction curve (Figure 7, j-events), representing the single integrin-ligand bonds and a second section at later stages of retraction curve, representing the membrane tethers (nanotubes) (Figure 7, t-events)\textsuperscript{103} (Figure 7). In addition the slope of the very last linear section of the extension curve illustrates the deformation of the cell body in response to applied forces by the cantilever (Figure 7). This parameter has been characterized as the contact stiffness (CS)\textsuperscript{104}, giving information about the elasticity of the cell body.

The main challenge in SCFS has been considered to be the interpretation of the data, because the recorded FD-curves illustrate the footprint of the cell adhesion as a whole process, which contains specific and unspecific cell adhesion\textsuperscript{103}. Since the setting parameters to record FD-curves are all adjustable in SCFS, the method can be used to investigate different aspects of cell adhesion. For example, by varying the applied force onto the cell during the interaction time, one can examine the effects of force dependent regulation of cell adhesion\textsuperscript{105}. Furthermore, the temporal dynamics of adhesion forces can be investigated by varying the interaction time between cells and substrate surfaces\textsuperscript{105}. Also, by coating substrate surfaces with certain ECM components and blocking unspecific integrin receptors, the strength of specific integrin-ligand bonds can be investigated\textsuperscript{104}.

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Figure 7: A force-displacement curve for characterizing cell-substrate adhesion. A cell is attached to a cantilever (1) and is extended towards the substrate surface by the piezo-electric-actuators until it reaches the surface (extension). The cantilever is then further pressed onto the cell body until the setpoint is reached (2). The cell is kept in contact with substrate surface for a certain interaction time and is finally separated from the substrate surface by being pulled away using the cantilever (retraction) (3). Cell adhesion forces are then measured using cantilever deflection (recorded by the segmented photodiode) and are shown as a function of the height of piezo-electric actuators (x-axis). The peak of the retraction curve (red curve) can be used as maximum force of detachment ($F_{\text{max}}$) to characterize the overall adhesion of the single cell. Furthermore, different j- and t-events are observed on the FD-curves, illustrating the separation of receptor-ligand bindings (j-events) and membrane tethers (t-events). (Modified from Aliuos et al. (2012a and 2012b)\textsuperscript{106,107})
1.6. The Objective of the Study

As it was mentioned before, the encapsulation of CI electrodes as a result of insertion trauma is initiated by the adsorption of ECM components and the adhesion of fibroblasts to the implant surface. Furthermore, it was discussed that physiochemical material surface alterations resulting in hydrophobic or hydrophilic properties of surfaces are suggested to affect the geometrical conformation of surface-adsorbed ECM components or to influence the binding forces between ECM components and material surfaces. Since both mechanisms have been shown to affect the adhesion of cells negatively, the use of such surface modifications seem to be very promising for the application in CIs in order to minimize the adhesion of fibroblasts to the surfaces of CI electrodes and electrode carriers. The objective of this study was to investigate the effects of hydrophobic and hydrophilic surface properties on the adhesion of fibroblasts. Furthermore, since nearly all studies on cell interactions with material surfaces have been done using qualitative methods, one of the goals of the study was to examine the feasibility of atomic force microscopic SCFS for quantitative characterization of fibroblast adhesion. We hypothesized that highly hydrophobic surfaces, generated by physical surface modification, reduce the adhesion of fibroblasts. The second hypothesis was that highly hydrophilic surfaces, generated by chemical surface alterations, suppress the adhesion of fibroblasts.

In the first part of the work and for physical modification of surfaces, titanium samples were first polished by Dr. Andreas Winkel. The next, using femtosecond-laser ablation different anti-wetting microstructures were generated on the sample surfaces and the wetting properties of the surfaces were examined by water contact angle measurements. Both procedures were done in Laser Zentrum Hannover eV. by Ms. Elena Fadeeva and Professor Dr. Boris Chichkov. The adhesion of NIH3T3 murine fibroblasts was investigated using SCFS, in order to examine the effects of the physically modified surfaces on the cell adhesion forces. Preparation of cells, SCFS-experiments, AFM-imaging, data processing, cell adhesion characterization, statistical analysis, and the documentation of the results were exclusively done by Pooyan Aliuos. Prof. Dr. Thomas Lenarz, Prof. Dr. Guenter Reuter, Dr. Uta Reich, Dr. Athanasia Warnecke, and Pooyan Aliuos contributed to the planning and interpretation of the results as well as to the documentation. In order to compare the observed adhesion forces with the proliferation of cells, cells were seeded on titanium samples and their proliferation as well as morphology was investigated by Mohammad Badar.
and Prof. Dr. Peter Paul Mueller. Both authors also contributed to the interpretation and documentation of the results regarding fibroblast proliferation. The gauged results of the first study period were discussed in a manuscript, which was submitted to the Journal of Biomedical Materials Research Part A on 25th of May 2012 and was accepted on 24th July 2012 for being published in the journal (Chapter 2).

In the second period of the study and to investigate the effects of chemically altered surfaces on cell adhesion forces, polymer coatings with hydrophilic surface properties were immobilized onto glass plates by Ms. Aromita Sen, Dr. Wibke Dempwolf, Mr. Christopher Hadler and Prof. Dr. Henning Menzel. Authors furthermore contributed to the documentation of the results regarding the characterization of surface properties (water contact angle measurements, NMR and ellipsometry). Cell-culturing, SCFS-experiments, AFM-imaging, data processing and interpretation, statistical analysis, and the documentation of the results were exclusively done by Pooyan Aliuos. Furthermore, Prof. Dr. Thomas Lenarz, Prof. Dr. Guenter Reuter, Dr. Uta Reich, Dr. Athanasia Warnecke, and Pooyan Aliuos contributed to the planning and interpretation of the results. The results were discussed in a manuscript, submitted to the J Biomed Mater Res A on 23rd of August 2012 (see chapter 2.2).

This thesis is presented in a chapter wise form and consists of two manuscripts (Chapters 2.1 and 2.2) with introductions, material and methods, results, discussions and conclusions, respectively. Furthermore, to give basic information about the relevance of the work in the medicine and cell biology an introduction is provided.

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Chapter 2: Results
Chapter 2.1


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Abstract
One goal in biomaterials research is to limit the formation of connective tissue around the implant. Anti-wetting surfaces are known to reduce ability of cells to adhere. Such surfaces can be achieved by special surface structures (lotus effect). Aim of the study was to investigate the feasibility for creating anti-wetting surface structures on titanium and to characterize their effect on initial cell adhesion and proliferation.

Titanium microstructures were generated using femtosecond- (fs-) laser pulses. Murine fibroblasts served as a model for connective tissue cells. Quantitative investigation of initial cell adhesion was performed using atomic force microscopy (AFM). Fluorescence microscopy was employed for the characterization of cell adhesion pattern, cell morphology and proliferation.

Water contact angle (WCA) measurements evinced anti-wetting properties of laser-structured surfaces. However, the WCA was decreased in serum-containing medium. Initial cell adhesion to micro-structured titanium was significantly promoted when compared to polished titanium. Microstructures did not influence cell proliferation on titanium surfaces. However, on titanium microstructures cells showed a flattened morphology and the cell orientation was biased according to the surface topography.

In conclusion, anti-wetting properties of surfaces were absent in the presence of serum and did not hinder adhesion and proliferation of NIH 3T3 fibroblasts.

Keywords: Cell-substrate adhesion, atomic force microscopy, femtosecond-laser, titanium microstructure, connective tissue growth
Chapter 2.2

Inhibition of Fibroblast Adhesion by Covalently Immobilized Protein repellent Polymer Coatings studied by Single Cell Force Spectroscopy

Inhibition of Fibroblast Adhesion by Covalently Immobilized Protein Repellent Polymer Coatings Studied by Single Cell Force Spectroscopy

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Abstract

Cochlea implants (CI) restore the hearing in patients with sensorineural hearing loss (SNHL) by electrical stimulation of the auditory nerve via an electrode array. The increase of the impedance at the electrode-tissue interface due to a postoperative connective tissue encapsulation leads to higher power consumption of the implants. Therefore, reduced adhesion and proliferation of connective tissue cells around the CI electrode array is of great clinical interest.

The adhesion of cells to substrate surfaces is mediated by extracellular matrix (ECM) proteins. Protein repellent polymers (PRP) are able to inhibit unspecific protein adsorption. Thus, a reduction of cell adhesion might be achieved by coating the electrode carriers with PRPs. The aim of this study was to investigate the effects of two different PRPs, poly(dimethylacrylamide) (PDMAA) and poly(2-ethyloxazoline) (PEtOx), on the strength and the temporal dynamics of the initial adhesion of fibroblasts.

Polymers were immobilized onto glass plates by a photochemical grafting onto method. Water contact angle (WCA) measurements proved hydrophilic surface properties of both PDMAA and PEtOx (45 ± 1° and 44 ± 1°, respectively). The adhesion strength of NIH3T3 fibroblasts after 5, 30 and 180 seconds of interaction with surfaces was investigated by using single cell force spectroscopy (SCFS). In comparison to glass surfaces, both polymers reduced the adhesion of fibroblasts significantly at all different interaction times and lower dynamic rates of adhesion were observed. Thus, both PDMAA and PEtOx represented anti-adhesive properties and can be used as implant coatings to reduce the unspecific ECM-mediated adhesion of fibroblasts to surfaces.

Keywords: Cell Adhesion, Single Cell Force Spectroscopy (SCFS), PDMAA, PEtOx, Cochlear Implants (CI)
1. Introduction

Inner hair cells, as the sensory cells of the hearing organ release neurotransmitters, which in turn lead to generation of post synaptic impulses in the first auditory neurons, the spiral ganglion neurons (SGN)\(^2,3\). The impulses are then directed to the brain, in order to be processed. Sensorineural hearing loss (SNHL) is characterized by the loss of hair cells, resulting in a degeneration of the neurites of SGNs\(^4,5\). Despite the degeneration of the neurites, cell bodies of SGNs and their projection to the brain stem survive for several years.

Cochlea implants (CI) restore the hearing of patients with SNHL by converting sound waves into electrical impulses, which stimulate the cell bodies of SGNs. Therefore, an array of active electrodes, enclosed in a soft and elastic silicone electrode carrier, is inserted into the scala tympani of the inner ear. As a result of insertion trauma and wound healing process, connective tissue encapsulates the electrodes and electrode carriers of CIs. Consequently, the impedance at electrode-tissue interface increases\(^25,108\). This phenomenon is responsible for higher power consumption of implants. Furthermore, a selective stimulation of neurons, which is important for differentiating between different sound frequencies, is interfered. Thus, for optimizing the performance of CIs, there is the need of novel material surfaces for electrodes as well as for electrode carriers in order to avoid the connective tissue encapsulation of the implant.

Adhesion of cells to surrounding surfaces (cell-substrate adhesion) is involved in the regulation of a wealth of cellular processes such as migration, proliferation, differentiation and wound healing\(^27,109\). For example, once adherent cells lose the attachment to the environment, they undergo apoptosis\(^27\). Cells adhere to surfaces by interacting with components of the extracellular matrix (ECM), which poses the natural environment of cells. The ECM is a three dimensional and complex meshwork consisting of a variety of molecules such as different glycoproteins, proteoglycans and non-matrix proteins including growth factors\(^30,110\). Cells are not only able to sense physiochemical properties of the ECM, but also to organize it by means of heterodimeric transmembrane receptors called integrins\(^111,112\). Integrins act as bi-directional transmitters, which from one side transduce the physiochemical signals of the surrounding tissue into the cells and from the other side change their affinity for adhesive peptides in response to forces that are applied by the cytoskeleton\(^26,34\). The extracellular domains of integrins bind to adhesive peptides of ECM components. Due
to this binding, the short intracellular tails of integrins undergo conformational changes, which lead to their linkage to the actin cytoskeleton through complexes of signalling and linking proteins, such as talin and vinculin\textsuperscript{26,113,114}. In mature adhesions such as focal or fibrillar adhesions, integrins build clusters and are properly connected to the actin cytoskeleton via the protein complexes\textsuperscript{34,111}. These adhesion sites are then used for example for the organization of the cytoskeleton and cell motility\textsuperscript{115}. A proper surface binding of ECM components is crucial for cell adhesion. Once cell adhesion mediating molecules of ECM such as fibronectins and vitronectins are weakly or not bound to surfaces, the adhesion of cells is interfered\textsuperscript{61}. The adsorption of ECM components to the surfaces depends on various physiochemical surface properties such as energy, electrical charge, polarity and wettability\textsuperscript{61}. For example, hydrophilic surfaces, characterized by high polar component of the surface energy, avoid the adsorption of cell adhesion molecules of ECM and, thus the adhesion of cells\textsuperscript{116}.

Different qualitative as well as quantitative methods for characterizing cell-substrate adhesion have been established and used\textsuperscript{98,117-120}. The atomic force microscopic (AFM) single cell force spectroscopy (SCFS) is the only quantitative method, which is able to characterize the adhesion of entire cells, because of its wide range of detectable forces (5 pN-100 nN)\textsuperscript{96}. After a single cell is attached to a functionalized cantilever, the mechanical sensor of the AFM, it is moved towards the sample until it reaches the surface. The cell is then kept in contact with the substrate surface by applying an adjustable amount of mechanical force onto it for a while (interaction time). The detachment force, which is applied to the cantilever during the retraction of the cell from the substrate surface, is finally measured and prospected as a force-displacement (FD) curve, illustrating the strength of the occurred force at the full range of cell displacement (Figure 1). More information on SCFS can be achieved elsewhere\textsuperscript{103,106}.

Poly(dimethylacrylamide) (PDMAA) and Poly(2-ethyl-2-oxazoline) (PEtOx) have been shown to provide hydrophilic and protein repellent surfaces\textsuperscript{68,121,122}. Furthermore, both polymers are reported as biocompatible and stable materials under biological conditions\textsuperscript{117,122-124}. Thus, their use as stable implant material coatings seems to be beneficial. Stable immobilization of polymer films onto material surfaces is crucial for ensuring the resistance against solvents and displacement reagents. Thus, covalently bound polymer films are desirable\textsuperscript{125}. Different methods have been developed for immobilizing thin polymer films on material surfaces\textsuperscript{126-128}. However,
most of these methods comprise complex and laborious synthetic steps. Additionally, the anchor groups are often very reactive and would interact with any other functional groups, which are present in the polymer films. Prucker et al. (1999) introduced a simple and effective grafting onto method, based on photochemical attachment of thin polymer films to solid surfaces. In this method, a photo-reactive benzophenone derivivate is attached to Si/SiO$_2$ surfaces by a silane anchor. A polymer film that covers the sample surface is covalently bound by the benzophenone moieties in a radical reaction, initiated by UV light illumination.

Even though CI electrode array consists of platinum electrodes and silicone electrode carrier, glass plates served as material to investigate the feasibility of polymer immobilization onto material surfaces by means of the “grafting onto” method. PDMAA and PETOx were immobilized onto glass plates and the success of coating process was examined by water contact angle (WCA) measurements. Furthermore the strength of adhesion of fibroblasts, as the main cellular part of the connective tissue, to hydrophilic protein repellent surfaces was measured using SCFS. We hypothesized that hydrophilic protein repellent surfaces diminish the adhesion forces of fibroblasts.

2. Materials and Methods

2.1. Chemicals

4-hydroxybenzophenone, allylbromide ($\geq 99\%$), azobisisobutronitrile (AiBN) (98\%), calcium hydride (CaH$_2$) (90-95\%), chlorodimethylsilane (96\%), dimethylacrylamide, diethylether (HPLC), methanol (HPLC), poly (2-ethyl oxazoline) (PETOx), potassium carbonate, Pt on activated charcoal, sodium hydroxide, sodium sulfate, toluene (HPLC) and triethylamine were purchased from Aldrich (Germany). Diethylether was dried over sodium overnight use and triethylamine was dried over CaH$_2$ overnight before use. All other chemicals were used as provided by the producers.

2.2. Synthesis of anchor and PDMAA

2.2.1. Anchor

The anchor (4-(3-Chlorodimethylsilyl)propyloxybenzophenone) was synthesized according to a method established by Prucker et al. (1999)$_{125}$. 

**Synthesis of 4-Allyloxybenzophenone**

4-hydroxybenzophenone (0.05 mol) and allylbromide (0.04 mol) were dissolved in 30 ml acetone in a 100 ml flask. Potassium carbonate (0.05 mol) was added and the reaction mixture was refluxed for 8 hrs at 75°C. After the mixture has cooled down to room temperature, water was added (30 ml). The mixture was extracted three times
with 25 ml diethyl ether and the combined organic phase was washed thrice with 10% NaOH solution. A colorless mixture was obtained. The organic phase was dried over Na₂SO₄. The solvent was evaporated until the crude product crystallized out and the product was recrystallized from methanol to yield 8.4 g (71%).

1H NMR: δ_H(CDCl₃, 400 MHz, δ in ppm): 4.6 (m, 2H, OCH₂), 5.35 (dd, 1H, H-1a), 5.45 (dd, 1H, H-1b), 6.1 (m, 1H, =CH-), 6.99 (m, 2H, arom. H), 7.45 (m, 2H, arom. H), 7.66 (m, 1H, arom. H), 7.8 (m, 2H, arom. H).

13C NMR: δ_C(CDCl₃, 100 MHz, δ in ppm): 69.0 (OCH₂), 114.2 (=CH-), 118.2 (H₂C=), 128.2, 129.7, 131.9, 132.5 (arom. C), 130.2 (C-11), 138.3 (C-7), 162.2 (C-4), 195.5 (C=O).

Synthesis of (4-(3-Chlorodimethylsilyl)propyloxybenzophenone)

In a 2-necked round bottomed flask fitted with a reflux condenser, 1.00 g (4.2 mmol) 4-allyloxybenzophenone and 5 mg Pt on activated charcoal were suspended in 10 ml dimethylchlorosilane under dry conditions and nitrogen atmosphere. The reaction mixture was refluxed overnight. The excess dimethylchlorosilane was removed under vacuum and the oily residue was dissolved in dry diethyl ether. The solution was filtered to remove the catalyst and the solvent was removed under vacuum. The obtained product was an oily liquid.

1H NMR: δ_H(CDCl₃, 400 MHz, δ in ppm): 0.34 (s, 6H, SiCH₃), 0.87 (m, 2H, SiCH₂), 1.84 (m, 2H, CH₂CH₂CH₂O), 3.92 (t, 2H, CH₂O), 6.7-7.9 (various m, 9H, arom. H)

13C NMR: δ_C(CDCl₃, 100 MHz, δ in ppm): 1.6 (SiCH₃), 15.2 (SiCH₂), 22.9 (CH₂CH₂CH₂), 69.9 (OCH₂), 128.2, 129.7, 131.9, 132.5 (arom. C), 130.2 (C-11), 138.3 (C-7), 162.2 (C-4), 195.5 (C=O) (Melting point = 85 °C).

2.2.2. Poly(dimethylacrylamide) (PDMAA)

A solution of dimethylacrylamide/methanol (3ml/7ml) was degassed in a stream of nitrogen for 30 minutes. The polymerization was started by adding 1 mol% (48 mg) of AiBN at 60°C. After one hour, the reaction mixture was concentrated until it was dry. The residue was dissolved in water and dialyzed (4000-6000 Dalton) in de-ionized water. Finally, the polymer was freeze-dried.

1H-NMR: δ_H(MeOH-d₄, 400 MHz, δ in ppm): 1.3-1.8 (2H), 2.7(1H), 2.9 (6H).

13C-NMR: δ_C(MeOH-d₄, 100 MHz, δ in ppm): 36.2, 36.3, 37.7 (C-1, C-2/C-4), 176.5(C-3).
2.3. Immobilization of anchor onto glass plates
Round glass plates with diameters of 23 mm were wiped with acetone. The plates were then cleaned with chloroform in an ultrasonic bath for 30 minutes. After the plates were dried under \(\text{N}_2\) stream, they were cleaned in a plasma cleaner for 30 min. They were then dipped in toluenic solution of 4-(3'chlorodimethylsilyl)benzophenone overnight at room temperature. This process was done in the presence of triethylamine, which acts as a catalyst and acid scavenger, under dry conditions and \(\text{N}_2\)-atmosphere. After ~15 hours the plates were washed thoroughly with chloroform. Contact angle measurements were performed to determine the wettability of anchor-coated surfaces.

2.4. Immobilization of polymer films onto anchor-coated glass plates
The polymer solutions (c = 10 mg/l) were spin-coated (spin coater, Specialty Coating Systems, Model P6700 Series, USA) on the plates. The spin-coated plates were then irradiated for 35 minutes with help of a UV lamp (200 W Hg-Lamp, Oriel Company, UK) with an intensity of 100 mW/cm\(^2\). In order to remove the infrared (IR) light, a water filter was used. To obtain UV radiation in the wavelength range of 360 ± 50 nm, a filter of type UG 1 manufactured by Spindler and Hoya GmbH (Goettingen, Germany) was used. Subsequently, the plates were washed in a Soxhlet apparatus for ~20 hrs using methanol to remove unbound polymer (extraction). Using contact angle measurements wetting properties of polymer films were characterized.

2.5. Water Contact Angle (WCA) Measurements
All contact angle measurements were performed using a Type G1 contact angle measuring instrument (Krüss GmbH, Germany). The measurements were carried out at room temperature using the tilted plate method\(^{129}\). The plates were tilted at an angle of 45\(^\circ\). A water drop was placed on the plate with the help of a syringe and the advancing and the receding angles were measured before the drop moved. Measurements were performed at five different spots on each sample surface.

2.6. NMR- Spectroscopy
The \(^1\text{H}\)-NMR and \(^{13}\text{C}\)-NMR spectra were recorded using two different spectrometers from Bruker (Type DRX 400 and AV 300, respectively) with the magnetic field strength of 9.4 T. The \(^1\text{H}\)-spectra were taken at a frequency of 400 MHz and the \(^{13}\text{C}\)-spectra at 100 MHz.

2.7. Surface Topography Characterization using AFM
Sample surface topographies were investigated via a NanoWizard II® AFM (JPK-Instruments AG; Berlin; Germany). Scan fields of 10*10 \(\mu\text{m}^2\) on all surface varieties
were probed in contact mode in distilled water using CSC21/AlBS cantilevers (MikroMash, Tallinn, Estonia) with a nominal spring constant of 2 N/m. Images were processed and prospected using the JPK data processing software (v. 3.4.18).

2.8. Cell Culture and Media

NIH 3T3 fibroblasts (LGC-Standards CRL-1658) were maintained in Dulbecco’s Modified Eagle’s Medium (DMEM, Biochrom AG, Germany) supplemented with 10% (v/v) fetal calf serum (FCS, Biochrom AG) and 1% (v/v) penicillin (10000 IE)/streptomycin (10000 µg/ml) (pen/strep, Biochrom AG). At 80% confluency, cells were trypsinized (Biochrom AG) using the standard protocol and resuspended into new culture flasks. Cells were passaged every third day. For cell adhesion experiments, serum free CO\textsubscript{2}-independent medium (Invitrogen GmbH; Germany) supplemented with 2%(v/v) FCS and 2%(v/v) stable glutamine (Biochrom AG) was used. This medium is denoted as experimental medium (EM) in this manuscript.

2.9. Single cell force spectroscopy (SCFS) experiments

All SCFS experiments were done at 37 °C in a BioCell® chamber (JPK). For ensuring the full separation of cells from sample surfaces, a CellHesion® stage (JPK) provided investigations with extended pulling length (z-length) up to 100 µm. The AFM was mounted on top of an Axio observer D1 inverse optical microscope (Zeiss, Jena, Germany). Samples were sterilized for 10 minutes in 70% ethanol, washed several times with distilled water, dried under gentle air convection and installed in the BioCell® chamber. The BioCell® was then filled with 600 µl of EM. A clean tipless silicon cantilever (Arrow-TL1, Nanoworld AG, Switzerland) with a nominal spring constant of 0.03 N/m was installed onto the AFM and was inserted gently into the BioCell. For ensuring a sufficient cell adhesive surface on cantilevers, these were functionalized with fibronectin from human plasma (Biochrom AG, Germany). In this study, cantilevers were calibrated prior to experiments using the thermal noise method\textsuperscript{102}. For more information on cantilever functionalization and calibration we refer to our previous study\textsuperscript{106}. Fibroblasts were washed with Hank’s saline solution, trypsinized and resuspended into the EM-filled BioCell. The final cell density of suspension was set to 5x10\textsuperscript{4}cells/ml and an amount of 100 µl of cell suspension was added to the BioCell® chamber. Then, an intact single cell was attached to the cantilever and was allowed to develop strong adhesion to the cantilever for at least 10 minutes, before the experiment began. More information on cell attachment and adhesion to cantilevers can be also gauged in our recent study\textsuperscript{106}. For all experiments,
we used a constant velocity of 2.5 μm/s for extension and retraction of cells. To ensure the attachment of cells to substrate surfaces over the whole period of interaction between cells and surfaces, the experiments were done in contact force mode and a constant force of 600 pN was applied onto the cells. Adhesion of cells to all samples was investigated at different interaction time intervals of 5, 30 and 180 seconds. For adequate statistics at least 13 single cells were probed on each sample surface. Cells were first allowed to adhere to surfaces for 5 seconds, then for 30 seconds and finally for 180 seconds. This process was repeated at least 5 times with each cell and at different spots on the surfaces. All generated data were processed using the JPK SPM software. For the quantification of cell adhesion, the peak of generated force-displacement (FD) curves was used and entitled as maximum force of detachment ($F_{\text{max}}$). Furthermore, the position of the peak on the z-axis was measured (Figure 1). For statistical analysis, two-way ANNOVA test was applied.

2.10. Cell diameter measurements

In order to be able to correlate the dimension of cells with their adhesion forces, the diameter of every single cell was measured. For this, photographs at magnifications of 100-fold of attached cells to cantilevers (Figure 2) were taken before an experiment started. Photographs were processed using ImageJ (1.6.0_31) by applying options of “scale” for calibrating the pixel size and “finding edges” for detecting the exact position of cell borders. The diameter of a cell was measured two times in two different orientations (Figure 2). The mean over these values determined the diameter of a single cell.

3. Results

3.1. Successful immobilization of polymers onto glass plates

In order to investigate the successful immobilization of the anchor, contact angle measurements were carried out. It was expected that the WCA on the glass surfaces increases in the presence of immobilized anchor monolayer. For anchor-immobilized glass plates, the obtained average advancing contact angle was 62 ± 1°. This value was larger than the one observed for untreated glass plates (25 ± 2°), indicating the change of the hydrophilic behavior of the surface to a hydrophobic one. Furthermore, ellipsometry was carried out on silicon wafers having a glass like silicon oxide layer. A thickness of 1.35 ± 0.3 nm was observed for the anchor layer. This result is in agreement with the previously reported values (thickness ~ 1 nm)\textsuperscript{125}. 

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In order to determine wetting properties of polymer coatings, after PDMAA and PEtOx were spin coated onto the anchor-coated glass surfaces, the WCA was measured. The WCA values for both PDMAA and PEtOx (45 ± 1° and 44 ± 1°, respectively) proved the successful immobilization of polymer films onto anchor-coated glass surfaces. Layer thickness of 10 nm for both PDMAA and PEtOx were determined, using ellipsometric measurements on silicon wafers as mentioned above. Moreover, AFM-images showed different surface topographies and roughness of sample surfaces for both polymers in comparison to glass and anchor-coated glass surfaces (Figure 3). The topography and roughness of glass (average roughness \( R_a \) ~ 170 picometers (pm)) surface was changed very slightly by the thin anchor film (\( R_a \) ~ 230 pm) (Figures 3A and 3B). On PDMAA coated surfaces, small holes (diameter ~ 100 nm, depth ~ 9 nm) indicated little uncoated areas (Figure 3C). Larger cavities (diameter ~ 600 nm, depth ~ 1.4 nm) were revealed on both PDMAA and PEtOx (Figures 3C and 3D).

3.2. The adhesion of fibroblasts was inhibited by PDMAA and PEtOx coatings

In order to study the dynamics of cell-surface adhesion, experiments were performed in different interaction time intervals (5, 30 and 180 seconds) (Figure 4). In general, the adhesion of fibroblasts to glass was significantly (\( p < 0.001 \)) stronger than to both polymer coatings (PDMAA and PEtOx). This was the case for all three different interaction times. However, very low detachment forces were revealed on PDMAA, the lowest rates of \( F_{\text{max}} \) were observed on PEtOx at all different interaction times (\( p < 0.001 \)).

Detachment forces of fibroblasts after 5 seconds of interaction to glass surfaces (409.90 ± 45.80 pN, Mean ± SEM) were promoted to 2070 ± 438.30 pN (Mean ± SEM) after 180 second. This equals an increase of cell adhesion forces by a factor of 5 after 175 seconds. On PDMAA, the detachment forces were increased from 5 seconds (93.48 ± 22.26 pN, Mean ± SEM) to 180 seconds (313.70 ± 61.30 pN, Mean ± SEM) by a factor of 3.37 and on PEtox by a factor of 2.15 from 5 seconds (69.78 ± 9.97 pN, Mean ± SEM) to 180 seconds (150.10 ± 28.20 pN, Mean ± SEM) (Figure 4).

Figure 5 shows FD-curves, which were recorded on different sample surfaces after 5, 30 and 180 seconds of interaction with cells. Differences in both detachment forces as well as in the number of force jumps (t- and j-events, Figure 1), indicating binding sites between cells and substrate surfaces\(^{103}\) were observed (Figure 5). Cells could strengthen their adhesion to glass surfaces over the time by assembling more binding sites (Figure 5, the upper row). This resulted in increased detachment forces by
a factor of 5 during 175 seconds (from 5 to 180 seconds) (Figures 4 and 5). Temporal changes in the number of binding sites between the cells and surfaces of PDMAA and PEtox were minor (Figure 5, the two lower rows) and the strength of adhesion was consequently increased by lower factors compared to glass surfaces (Figures 4).

In order to exclude errors in this study, the strength of cell adhesion to anchor-coated glass plates was examined. Compared to glass, PDMAA and PEtox, higher detachment forces were revealed on anchor-bound surfaces (data not shown here).

3.3. Detachment forces of fibroblasts does not depend on cell size
To study the dependency of $F_{\text{max}}$ from the cell size, the detachment forces of single cells at different interaction times were visualized on a diagram as the function of cell diameter. We revealed cell sizes from 12.58 µm to 19.04 µm and $F_{\text{max}}$ from 844.33 pN to 7617 pN on glass. On glass surfaces (Figure 6) as well as on both polymers (data not shown), the $F_{\text{max}}$ was found to be independent from the cell size at all different interaction times (5, 30 and 180 seconds) (Figure 6).

3.4. Higher detachment forces sum up at higher positions on the height-axis
In this study, we hypothesized that the summing up of the maximum force of detachment in cells with stronger adhesion would take more time and therefore the position of the maximum peak of the curve should take place at a higher level on the vertical axis (position of the piezo-electric actuators) as the cells are retracted from the surface. For this $F_{\text{max}}$ of cells were visualized as a function of piezo-height at the point of occurrence of $F_{\text{max}}$. A linear regression was fitted to the data points to examine the correlation between $F_{\text{max}}$ and its position (Figure 7). The slope ($s$) of the fitted regression ($3.53 \pm 0.14$, Mean ± SD) to the data revealed on glass was significantly different from zero ($p < 0.0001$), indicating the positive correlation of the strength of $F_{\text{max}}$ with the position of its occurrence on the height axis. Thus, larger detachment forces occurred at higher levels of the piezo height (Figure 7). This manner could not be confirmed by the observations on PDMAA and PEtox ($s = -0.17 \pm 0.11$ and $0.06 \pm 0.03$, respectively, data not shown).

4. Discussion
This study presents quantitative results regarding the strength of fibroblast adhesion to two hydrophilic polymer surfaces (PDMAA and PEtox), which were covalently immobilized onto glass surfaces. The successful immobilization of PDMAA and PEtox onto glass surfaces as a model system for Si/SiOH surfaces was proved by water contact angle measurements as well as elipsometric and atomic force microscopic
investigations. We then evaluated the strength of fibroblast adhesion to the generated polymer coatings. Furthermore, the temporal dynamics of the cellular adhesion to polymer surfaces was investigated and compared with that to glass surfaces.

In accordance to another study\textsuperscript{125}, ellipsometric results revealed a layer thickness of \(~ 1.4\) nm for the anchor. Assuming that all anchor molecules are assembled on the surface in all-trans form (Figure 8), the layer thickness is expected not to be more than \(1.5\) nm, which is the length of the anchor molecule. Thus, it can be concluded that a monolayer of the anchor was successfully immobilized onto glass surfaces. By immobilizing PDMAA and PEtOx onto anchor-coated glass plates (WCA = 62°), the contact angle of the water drop was decreased to lower values (WCA = 45° and 44°, respectively), indicating more hydrophilic surface properties for both polymer coatings. Interestingly, the AFM-images of PDMAA-surfaces showed few little holes (diameter \(~ 100\) nm, depth \(~9\) nm) (Figure 3C). Taking the measured layer thickness of \(~ 10\) nm (PDMAA and anchor) into account, this observation indicates a not completely homogenous polymer coating over the anchor monolayer. Moreover, some cavities with larger diameters (\(~ 600\) nm) but lower depths (\(~ 1.4\) nm) appeared on both PDMAA- and PEtOx-surfaces (Figures 3C and 3D). Therefore, a not very homogeneous surface coating can be concluded. Nevertheless, WCA as well as AFM measurements proved the successful polymer immobilization.

As mentioned in the introduction, both PDMAA and PEtOx have been termed as protein repellent and biocompatible polymers in several studies, making them candidates for implant materials or implant surface coatings. For example, Petersen and Biesalski (2011) investigated the specific adhesion of human fibroblasts to a fibronectin-derived peptide-ligand and used PDMAA as a protein-resistant background to avoid unspecific adhesion of human fibroblasts\textsuperscript{117}. Protein repellent properties of PDMAA were also used earlier by Loschonsky et al. (2008) to protect surfaces from unspecific protein adsorption\textsuperscript{130}. Consequently, the adhesion of human skin fibroblasts to the background was inhibited. In another study, the adsorption of fibronectin and the adhesion of human aortic endothelial cells adhesion to PDMAA were examined\textsuperscript{121}. Authors reported that PDMAA prevented the adsorption of fibronectin and consequently the adhesion of cells to the sample surfaces. In addition to their protein repellent surface properties, both PDMAA and PEtOx have been demonstrated to be non-toxic, biocompatible and stable under biological conditions\textsuperscript{117,122-124}. For example, Bauer et al. (2012) studied the cytotoxicity and blood compatibility of PEtOx of
different molecular masses at different concentrations using L929 murine fibroblasts and erythrocytes. PEtOx was reported to be well tolerated by both cell types. Furthermore, the authors underlined beneficial physiochemical properties of PEtOx, such as high stability in comparison to poly(ethyleneglycol) (PEG), highlighting the polymer for implant surface coatings. The electrode carriers of CIs consist of very elastic and soft medical silicone, which facilitates a soft insertion of the electrode carrier into the spiral-shaped cochlea. Therefore, there is the need to coat the implant surface without changing its elasticity to maintain a soft insertion. Thus, stable covalently surface-bond thin polymer monolayers on the implant surfaces would be promising in this context. Therefore, the feasibility to produce such surfaces and the effects of generated surfaces on cell adhesion, as the initiator of further cellular cues, such as spreading and proliferation were the aims of this study.

Figure 4 shows how surface properties of different samples affected the detachment forces of fibroblasts at different interaction times. The glass surfaces, as it was also shown earlier in our recent study, introduced an adhesive surface. The detachment of fibroblasts after three minutes of interaction with glass surfaces was increased by a factor of 5 compared to that after 5 s of interaction, indicating the dynamic development of the adhesion forces. Different mechanisms could be involved in this phenomenon. For example, it is known that focal adhesions strengthen by recruiting vinculin and become more stable. Tan et al. (2010) investigated the recruitment of vinculin to focal adhesions in their assembly and disassembly phases. They showed that vinculin was recruited in ~13 seconds to nascent focal adhesions, which were assembled due to shear stress. They furthermore observed that focal adhesions are assembled in less than one minute in the presence of mechanical stimulus. Nevertheless, the whole process of adhesion site assembling would only develop if integrins can build clusters.

In comparison to glass surfaces, both PDMAA and PEtOx showed highly anti-adhesive surfaces (Figure 4). The forces, which have been generated even after 180 s of cell interactions with PDMAA and PEtOx surfaces, are smaller (313.7 ± 61.4 and 103.7 ± 20.8 pN, respectively; Mean ± SEM) than those observed on glass surfaces after 180 s (2070 ± 438.3 pN). Interestingly, the adhesion of cells to glass surfaces was even after 5 seconds of interaction (405.9 ± 45.8 pN) stronger than to both polymers after 180s. PEtOx presented the most anti-adhesive surface and in nearly all the cases, only a few binding sites between cells and substrates could be observed (Figure 5). In a
recent study, we demonstrated results regarding the strength of adhesion forces to glass surfaces after an interaction time of 30 s. Since we used serum-free medium, the generated forces should have occurred because cells attached to the surfaces using their cytoplasm-bound hyaluronan or by producing their own extracellular matrix by secreting proteins onto the surfaces, as it has been shown in other studies. Consequently and since the experiments of the present study have been done in serum-containing medium (2%), the results indicate that PDMAA and PEtOx coatings not only inhibit the adsorption of ECM proteins but also the hyaluronan-mediated cell adhesion.

As it has been hypothesized, the proteins of ECM are bound to highly hydrophilic surfaces by weak forces. Furthermore, the development of focal adhesions is highly dependent on sufficient mechanical forces, acting at extracellular and intracellular tails of integrin heterodimers. Thus, the integrin-mediated adhesion of fibroblasts to the hydrophilic polymer surfaces in this study is suggested to be inhibited by three different mechanisms. First, as a result of weak binding of ECM proteins to the hydrophilic polymer coatings, the mechanical forces that were generated at the extracellular integrin domains were not sufficient to unfold the intracellular tails. Consequently, integrin clustering and binding to actin-linking protein complexes was suppressed leading to very weak detachment forces of cells. Second, some binding of adhesion receptors to weakly bound ECM molecules to the polymer coatings has occurred due to the mechanical stress that was generated by the cantilever. Nevertheless, as cells were retracted from the sample surfaces some faint detachment forces were generated due to separation of weakly bound ECM molecules from the hydrophilic surfaces and not because of the rupturing of receptor-ligand bonds. Third and very likely, while the polymers themselves were totally inert against the protein adsorption, some little amount of proteins were adsorbed onto the little non-coated areas (Figure 3C), resulting in a few integrin-ligand connections and consequently very low detachment forces. Also the possibility of combinations of all three effects is not excluded.

The strength of adhesion of 3T3NIH fibroblasts, observed in this study was independent from their size. This effect has been also reported by earlier studies (e.g. by Coman 1944). We suggest that the changes in the detachment forces, revealed in different single cells depend rather on the activity and the cell cycle dynamics of those examined cells. For example, Song et al. (2005) showed that the adhesion of human
hepatoma cells to endothelial cells was much weaker in the G-phase than in the S-phase of the cell cycle. Although this observation was gauged by investigating cell-cell adhesion, similar effects have been suggested to affect the cell-substrate adhesion of primary human osteoblasts\textsuperscript{136}. 

As cells are retracted from substrate surfaces by being moved via the cantilevers (driven by piezo-electric actuators) over the vertical axis, the elastic cell membrane is subjected to tension. This tension increases with a rise in adhesion strength, leading to mechanical deformation of the cell body after longer interaction times\textsuperscript{103}. Consequently, cells with stronger adhesion to underlying surfaces need to be dragged longer by the cantilever, until the adhesive bonds are separated. This explains why stronger detachment forces occurred at higher positions on the height-axis (Figure 7). In addition, there is cytoskeletal contact between different adhesion sites and between them and the cell core\textsuperscript{37}. Thus, as the tension in cell membrane increases, there is the possibility that the connections in the cytoskeleton break before the extracellular domains of adhesion receptors are separated from their ECM ligands. Taking this into account, it is not excluded that the detected forces after 180 seconds of interaction include some intracellular cohesion forces.

5. Conclusion

Two different polymer films with hydrophilic surface properties were successfully immobilized onto glass surfaces. The adhesion of 3T3NIH murine fibroblasts to both polymeric surfaces was investigated qualitatively using SCFS. In comparison to glass surfaces, the adhesion of cells to hydrophilic surfaces of PDMAA and PEtOx was significantly lowered. The results indicate that the adhesion of cells to hydrophilic surfaces was diminished because of the poor attachment of ECM components to the surfaces. In conclusion, the anti-adhesiveness of both polymer coatings was proved. PDMAA and PEtOx promise not only good biocompatibility and stability under biological conditions, but also inertness against unspecific protein adsorption resulting in anti-adhesive properties for fibroblasts. Thus, the covalent immobilization of these polymers onto implant material surfaces, would lead to the inhibition of unspecific protein adsorption and undesired cell adhesion, while keeping the physical material properties such as elasticity untouched. In further studies the feasibility of coating implant material surfaces should be proved. Furthermore the response of living organism to the polymeric surfaces should be investigated in long time \textit{in vivo} studies.
6. Acknowledgment

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7. Figures

![Image of a force-displacement (FD) curve and used parameters for characterizing cell adhesion.](image)

**Figure 1: Illustration of a force-displacement (FD) curve and used parameters for characterizing cell adhesion.** A cantilever with an attached cell was moved towards the substrate (extension) until it reached its surface and a constant force (setpoint) was applied to maintain the contact between the cell and substrate surface over the interaction time. As the cell was retracted from the underlying surface (retraction), cell-surface bindings (little arrows) were detached and the cantilever was subjected to deflections by the applied mechanical forces. In this study the peak of the retraction curve (the red curve) was used to investigate detachment forces of cells ($F_{\text{max}}$). Furthermore the position of the peak on the x-axis (height-axis) was correlated with the strength of $F_{\text{max}}$. 
Figure 2: Processed photograph of an attached single cell to a cantilever for measuring its diameter. After a cell was captured by a cantilever it was allowed to adhere there for ~10 minutes. Afterwards, photographs with a magnification of 100X were taken. They were then processed using “finding edges” of ImageJ and the diameter of the cell was measured in two different orientations, indicated by white crossed lines. The diameter of a cell was the mean over both measured diameters. The average diameter of the whole cells measured in this study (n = 38) was 15.47 ± 2.02 (Mean ± SD).
Figure 3: AFM-height images of glass surface (A), anchor-coated glass plate surface (B), PDMAA-coated surface (C) and PEtOx-coated surface (D). The immobilization of glass surfaces with anchor led to minor changes of the surface topography and roughness (compare A and B). On PDMAA little holes (Figure C, white arrows and cross section) with diameters of ~ 100 nm and depths of 8-9 nm were observed, indicating areas, which have not been covered with the polymer. Also some holes with larger diameters (< 1 µm) but lower depth (~ 1.4 nm) were observed on both PDMAA and PEtOx surfaces (Figure D, white arrows and cross section).
Figure 4: Compared to glass, the strength of fibroblast adhesion was significantly lowered by PDMAA and PEtOx coatings. Detachment forces of fibroblasts (y-axis) to glass, PDMAA and PEtOx are visualized at different time intervals of interaction between cells and surfaces (x-axis). The adhesion of fibroblasts was decreased significantly ($p < 0.001$; two-way ANOVA) by using PDMAA and PEtOx at all different interaction times. PEtOx showed the most anti-adhesive surface properties in comparison to both glass and PDMAA ($p < 0.001$). After 180 s of interaction with surfaces the adhesion of fibroblasts to glass was promoted by a factor of 5, whereas the adhesion of cells to PDMAA and PEtOx was increased by lower factors (3.37 and 2.15 respectively).
Figure 5: The temporal development of cell adhesion was inhibited by PDMAA and PEtOx as a result of suppressing receptor-ligand binding sites. FD-curves showed different manner at different time points (5, 30 and 180 seconds) on all investigated sample surfaces (Glass, PDMAA and PEtOx). While the adhesion of cells to glass was promoted strongly and cells showed a temporal development of binding cites (upper row), the adhesion of cells to both polymer coatings showed minor changes (two lower rows).
Figure 6: Adhesion of fibroblasts to surfaces is independent from their size. The detachment forces of fibroblasts (n=15) observed on glass surfaces after different interaction times (5, 30 and 180 seconds) are shown as a function of cell diameters in adherent state at cantilevers (Figure 2). Because no correlation was observed between occurred forces and the size of cells, the dependency of $F_{\text{max}}$ from cell diameters was excluded.
Figure 7: Higher detachment forces of fibroblasts to adhesive glass surfaces occurred at higher levels on z-axis. To investigate if detachment forces correlate with the position of piezos at which $F_{\text{max}}$ occurred, we visualized $F_{\text{max}}$ as a function of piezo height. A linear regression was then fitted to the data (straight line). The slope of the regression line ($s = 3.52 \pm 0.29$, Mean ± SD) was significantly different from zero ($p < 0.0001$), indicating that dependence of $F_{\text{max}}$ from the position of its appearance on z-axis.

Figure 8: Schematic drawing of anchor (4-(3’ Chlorodimethylsilyl)-benzophenone) immobilization onto glass surfaces. Figure shows the assembly of anchor molecules on glass surfaces in an all-trans form.
3. Discussion

In this thesis the adhesion of NIH3T3 murine fibroblasts to physically and chemically modified surfaces was investigated using the quantitative method of single cell force spectroscopy (SCFS). The goal of the study was to examine the feasibility of SCFS for quantitative characterization of cell-substrate adhesion. Furthermore, the method was used to investigate the effects of physiochemical surface properties on cell adhesion. It was hypothesized that hydrophobic (anti-wetting) and hydrophilic (wetting) surfaces are both able to decrease the adhesion of cells. In order to generate anti-wetting microstructures on titanium as a biocompatible material, femtosecond- (fs-) laser ablation was used. For chemical modification of surfaces, glass plates were coated with two different polymers (PDMAA and PEtOx) with hydrophilic surface properties. Since both the results concerning physically and chemically altered surfaces has been discussed in earlier chapters (Chapters 2.1 and 2.2), a short repetition of those discussions and a general discussion are provided in this section of the thesis.

3.1 Correlation between Detachment Forces and Physical Surface Properties

As it was discussed in chapter 2.1, fs-laser ablation is a method, which provides surface structuring without changing chemical composition of surfaces. Using different fs-laser fluences two different surface topographies, both with similar anti-wetting properties (WCA ~ 162°) (Chapter 2.1, Table 1), but with different roughness and different spatial organization of local microstructures (Chapter 2.1, Table 1 and Figures 3 and 4) were generated. Both micro-structured surfaces showed local structures, which were the same size as the investigated cells. Glass and polished titanium surfaces, both with similar wetting properties (WCA ~72°) (Chapter 2.1, Table 1), but with different roughness (Chapter 2, Table 1) served as control surfaces.

The adhesion of fibroblasts after 30 seconds of interaction to all four different surface varieties was investigated under the same physiological (serum free medium) and mechanical (setpoint = 800 pN, extension/retraction velocity = 2.5 m/s) conditions. Results showed that the adhesion of cells to micro-structured surfaces was significantly stronger than to polished titanium surfaces. Since the strength of cell-surface adhesion has been shown to correlate positively with the cell-surface contact area and both microstructures provided cavities with the same size as the cells (Chapter 2.1, Figures 3 and 4), it was concluded that an increase in the cell-surface contact area was
responsible for higher adhesion forces. Taking into account that in SCFS the cell is pressed slightly onto the surface by the cantilever leading to a deformation of the cell body\textsuperscript{103}, the cell-surface contact area is suggested to be further increased.

Changes in detachment forces of cells, revealed on glass and polished titanium were suggested to be based on other surface properties than surface wettability, because both surfaces showed the same wetting properties (Chapter 2.1, Table 1). As mentioned in the introduction (Chapter 1.4), the adsorption of ECM components as well as the adhesion of cells to surfaces depends not only on surface wettability, but also on the electrostatic charges of substrate surfaces\textsuperscript{61}. Since the electrostatic charges of glass and titanium surfaces could vary, it is suggested that this parameter determined the differences in detachment forces. An interesting task here would be the determination of surface electrostatic charges and the correlation of detachment forces with the electrostatic charges of material surfaces.

3.2 Correlation between Detachment Forces and Chemical Surface Properties

In this study besides the surface topography (Chapter 2.1)\textsuperscript{106}, also the chemical modification of surfaces (Chapter 2.2)\textsuperscript{107} affected the strength of cell adhesion. For chemical modification of substrate surfaces, two polymers (PDMAA and PETOx) (Chapter 2.2) that are known to evoke protein repellent surfaces\textsuperscript{68,121,122} as well as good biocompatibility and stability\textsuperscript{117,122,124} were immobilized onto glass surfaces using a grafting onto method. As mentioned before, the photochemical grafting onto method leads to covalent immobilization of thin polymer layers onto substrate surfaces and provides therefore stable surface coatings\textsuperscript{125}. The strength of fibroblast adhesion to both polymers as well as to glass surfaces and anchor-covered surfaces was investigated by applying SCFS under the same experimental conditions (2\% serum-containing medium, setpoint = 600 pN and extension/retraction velocity = 2.5 \(\mu\)m/s). The hydrophilic properties of substrate surfaces were then correlated with the measured adhesion forces. Moreover, the temporal development of adhesion forces was investigated by measuring the strength of the cell adhesion at different interaction times (5, 30 and 180 seconds) on all different substrate surfaces (Chapter 2.2)\textsuperscript{107}.

The hydrophilic properties of both polymers were proved by low water contact angles (44 ± 1° on PETOx and 45 ± 1° on PDMAA). The much lower WCA, measured on glass surfaces in chapter 2.2 (25 ± 2°) compared to that measured on glass surfaces
in chapter 2.1 (72°), is probably the result of using different glass cleaning processes in both chapters (alcohol and chloroform in ultrasonic bath, for chapters 2.1 and 2.2, respectively). Though, the SCFS experiments in chapter 2.2 were performed on glass plates, which were cleaned using alcohol (see chapters 2.2, Materials and Methods)\textsuperscript{107}. Thus, it is suggested that the cells adhered to glass surfaces with probably the same wetting properties like in chapter 2.1 (WCA = 72°).

In comparison to physical surface modification\textsuperscript{106}, the coating of glass substrates with polymers led to minor changes in the surface topography and roughness\textsuperscript{107} (Chapter 2.2, Figure 3). Since also the changes in the cell size were negligible (diameter = 15.99 ± 2.1 µm, 15.21 ± 2.1 µm and 15.14 ± 1.9 µm for glass, PEtOx and PDMAA, respectively), it is assumed that the cell-surface contact area in the experiments, performed on all different substrate surfaces was kept constant. Thus, the alterations in the detachment forces of fibroblasts have been exclusively generated by other mechanisms than by varying the cell-surface contact area.

Compared to glass surfaces as well as to anchor-coated surfaces (data not shown), the strength of fibroblasts adhesion to PEtOx and PDMAA at all different interaction times was significantly decreased (Chapter 2.2, Figure 4) and PEtOx showed the most anti-adhesive surface properties\textsuperscript{107}. While fibroblasts strengthened their adhesion to glass surfaces by a factor of 5 (from 5 to 180 s of interaction), the adhesion of cells to PEtOx and PDMAA after the same duration was increased by lower factors (2.15 and 3.37, respectively)\textsuperscript{107}. Considering the fact that the adhesion of cells after 180 second of interaction with PEtOx and PDMAA was significantly weaker than the strength of fibroblast adhesion to glass surfaces after only 5 seconds of cell-surface interaction, it can be concluded that both polymer coatings provided anti-adhesive surfaces\textsuperscript{107}.

The shape of the FD-curves changed in an interaction time-dependent manner, indicating that the adhesion of cells to adhesive glass substrates increased rapidly and was associated with an increase in the number of cell-substrate bindings (Chapter 2.2, Figure 5), as it was assumed and has been shown in other studies\textsuperscript{103}. In contrast, the development in the number of cell-substrate bindings on both PEtOx and PDMAA was suppressed and only few binding sites were developed even after 180 seconds of interaction between cells and surfaces (Chapter 2.2, Figure 5)\textsuperscript{107}. It is suggested that the hydrophilic surfaces led to an inhibition of cell adhesion by three possible mechanisms. First, the adsorbed ECM components were bound to the hydrophilic surfaces in a very
elastic form, as it is for example suggested by Bacakova et al. (2011)\textsuperscript{61}, and thus the mechanical forces acting at the interface between receptors and their ligands were insufficient to initiate the cascades for integrin clustering and building adhesion sites. As mentioned in the introduction, integrins are bi-directional signalling molecules, which mediate a mechanosensitive behaviour of binding sites and consequently the adhesion sites, such as focal complexes and focal adhesions\textsuperscript{26,47}. Furthermore, it has been shown that the assembly of cell-substrate adhesion depends on external and internal forces acting at the adhesion sites\textsuperscript{47,53}. Thus, if the ECM is too soft and elastic, the adhesion of cells would fail, as it has been shown in earlier studies\textsuperscript{61}. A second possibility is that even though the ECM components were organized very softly over the substrate surfaces, it could come to some receptor-ligand bindings due to the mechanical stress applied by the cantilever. Since, the binding between hydrophilic surfaces and ECM components (adhesive ligands) is suggested to be performed by very weak forces\textsuperscript{61}, as the cells were retracted from the hydrophilic surfaces, weak detachment forces as a result of rupturing the weak bonds between surfaces and ECM components and not due to the separation of receptor-ligand bonds occurred. Third and most likely, as a result of the adsorption of few ECM molecules to the anchor immobilized areas, which were not completely coated with PDMAA or PEtOx (Chapter 2.2, Figures 3C and 3D), some binding between adhesion receptors and their ligands occurred. These few binding sites contributed to the observed weak detachment forces of cells. The higher possibility of the third mechanism arises, since anchor-coated glass surfaces showed the most adhesive surfaces in this study (Chapter 2.2)\textsuperscript{107}. Still, combinations of all three mechanisms are not unlikely.

3.3 Effects of Physiochemical Surface Properties on Cell-Substrate Adhesion

The cell-substrate adhesion is mediated by a big variety of ECM components, such as glycoproteins, proteoglycans and polysaccharides\textsuperscript{30,31} and different specific and non-specific cell adhesion receptors such as integrins, Ig-superfamily and hyaluronan\textsuperscript{31,32}. Physiochemical properties of substrate surfaces affect the adhesion of cells by different mechanisms, such as by changing the geometrical and spatial distribution of ECM components over the substrate surface, by altering the cell-surface contact area and by influencing the absolute number of adsorbed ECM components or their binding strength to the substrate surfaces\textsuperscript{61,106,131,137}. 

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It has been suggested that hydrophobic surfaces lead to the absorbance of ECM molecules in a denatured geometrical formation, which is inappropriate to be bound by cell-adhesion receptors\textsuperscript{61}. To examine the effect of the surface geometry on cell-substrate adhesion, serum was excluded from the medium in the first section of this study (Chapter 2.1)\textsuperscript{106}. Nevertheless, higher adhesion forces were revealed on both generated microstructures (Mic1 and Mic2) than on polished titanium (chapter 2.1). Because the dimensions of the microstructures were in the same range as cell sizes, it was concluded that larger cell-surface contact areas led to higher adhesion forces\textsuperscript{106}. Since serum-free medium was used, an interesting question is, if cells adhered only by the membrane-bound hyaluronan or they developed their own extracellular matrices by secreting some little amounts of ECM proteins, such as fibronectin, as it has been shown in other studies\textsuperscript{133,138}. A further possibility is that, after cells were trypsinized and suspended they still carried some ECM proteins at their periphery and could attach to the surfaces after these proteins were adsorbed to substrate surfaces. Still, it was shown that the effect of surface morphology was greater than that of the anti-wetting properties of the physically altered surfaces. This proved not only by SCFS results, but also by the investigations regarding the proliferation of cells on different titanium surfaces. Since fibroblasts showed similar proliferation rates on both micro-structured and polished titanium but changed morphology on the microstructures, it was concluded that could grow in a normal manner by adapting their shape to the surface structures (Chapter 2.1).

Since the glue-like hyaluronan coats the surface of cells without the need of being activated\textsuperscript{31}, the presence of hyaluronan-mediated cell adhesion in both sections of the study (Chapters 2.1 and 2.2) is assumed. But because the used medium in the second part of the study (Chapter 2.2) contained some serum (2% v/v), the probability of the receptor- (integrin-) mediated adhesion in that section of the study is higher than in chapter 2.1. Furthermore, since the assembly/disassembly of focal adhesions (mature form of the focal complexes) occurs within less than one minute (32.2 ± 2.2 s for assembly and 60.5 ± 6.0 s for disassembly)\textsuperscript{46} under mechanical stress, the occurrence of integrin-mediated adhesion in this study is ensured, because cells were subjected to mechanical stress by the cantilevers and interacted with substrate surfaces for 30 seconds in the first part and for 5, 30 and 180 seconds in the second part of the study. Taking all this into account, it is concluded that hydrophilic polymer coatings (PDMAA
and PEtOx) diminished not only the hyaluronan-mediated but also the integrin-mediated adhesion of fibroblasts\(^{107}\).

Additionally to the detachment forces of single cells, also the contact stiffness was measured in both sections (Chapter 2.1, Figure 7 and Chapter 2.2, results not shown). In both cases and in agreement with another study performed on Hela cells\(^{104}\), the adhesion forces of single fibroblasts were totally independent from their stiffness. Considering a cell as a deformable and elastic body\(^{103,105}\), which is faced to deformations by being pressed via the cantilever, and if the glue-like hyaluronan-mediated adhesion would be more dominant than the integrin-mediated adhesion in the first seconds of cell-surface interaction, an increase of cell-surface contact area would lead to higher detachment forces. Consequently, since cells with lower stiffness undergo higher cell body deformations in response to mechanical pressure (by cantilever) and flatten more than stiffer cells, the strength of their adhesion should have been higher than that of stiffer cells. Thus, as the strength of adhesion of fibroblasts was independent from their stiffness, it is likely that the active receptor-mediated adhesion played a more dominant role than the passive hyaluronan-mediated adhesion. This is also assumed from the results on the correlation between cell size and detachment forces, since both parameters were shown not to correlate (see chapter 2.2, discussion). Moreover, the more dominant influence of receptor-ligand adhesion was also confirmed by the changes in the shape of FD-curves, since more receptor-ligand bindings occurred with increased interaction time (Chapter 2.2, Figure 5).

Friedrichs et al. (2010)\(^{104}\) showed that the functionalization of cantilevers with different ECM components affects the results of SCFS. For example, it was shown that detachment forces of Hela cells, which were attached to fibronectin-functionalized cantilevers was reduced if the cells were allowed to interact with fibronectin-coated substrate surfaces\(^{104}\). It was concluded that the fibronectin-specific integrins (\(\alpha5\beta1\)) mediated the adhesion of cells to the cantilevers and thus, contributed less to the adhesion of cells to the underlying fibronectin-coated surfaces. Therefore and since cantilevers were functionalized with fibronectin, it is considered that the results of this study show a less fibronectin dependent cell-substrate adhesion. In addition, since the application of external forces to the cell surface strengthens the focal adhesions (e.g. by initiating an over expression of vinculin) (more information in chapter 1.3 and ref. [45]), it is suggested that the absolute values of detachment forces detected in this study may be overestimated. Nevertheless, because all of the experiments have been
performed under the same experimental conditions the method was well usable to compare the adhesion of cells to different surface modifications.

Even though SCFS is toilsome and time lasting, it provides well defined FD-curves with high resolution of both forces and displacements. In this study it was shown that the method can be well applied in different ways to study different aspects of cell-substrate adhesion. For example, the effects of increased cell-surface contact area on adhesion of single fibroblasts was detected (Chapter 2.1). In addition, effects of anti-adhesive hydrophilic polymer surfaces on fibroblasts adhesion was successfully investigated and compared with the effects of adhesive glass surfaces on cell adhesion (Chapter 2.2). Also the temporal development of fibroblasts adhesion to adhesive and anti-adhesive surfaces was examined, by varying the cell-surface interaction time.

Besides the detachment forces of single fibroblasts, also their stiffness was quantified and both parameters were shown to be independent from each other. Furthermore, using additional methods, such as fluorescent microscopy and conventional light microscopy other important parameters such as the proliferation of cells on physically altered surfaces or the dimension of single examined cells were correlated with their detachment forces. For example, it was found that the size of fibroblasts is not the determining parameter for the strength of their adhesion. Taken all this together, the feasibility of SCFS for characterizing the adhesion of single cells to physically and chemically modified substrate surfaces was proved. Still, the interpretation of the recorded FD-curves seems to be challenging because they illustrate the whole process of adhesion without differentiating between specific and non-specific adhesion. Nevertheless, since the adhesion of cells to their natural environment in the body is also mediated by specific and non-specific adhesions, the method is well applicable for investigating the absolute strength of cell-substrate adhesion by using the maximum force of detachment as a parameter, as it is done in this study.

4. Summary
To restore the hearing in patients with profound hearing loss cochlear implants are used to stimulate the auditory nerve using an array of active platinum electrodes, embedded in a soft silicone electrode carrier. The insertion of the electrode array into the scala tympani causes an acute wound, resulting in the encapsulation of the implant with connective tissue. Consequently, the impedance at the electrode-nerve interface is increased and the selective stimulation of nerve fibres is interfered. The adhesion of
cells to their environment regulates diverse cellular cues such as proliferation. Furthermore, the strength of cell adhesion to implant surfaces depends on the physiochemical surface properties. Therefore, the implant surfaces can be modulated to alter the strength of cell adhesion. Since, qualitative methods for characterizing cell adhesion, such as immunocytochemistry do not give information about the strength of interacting forces at the cell-surface interface (e.g. hydrophilic/hydrophobic or molecular forces), the objective of this study was the quantitative characterization of cell adhesion to physically and chemically modified surfaces, in order to investigate the mechanisms that are involved in the generation of adhesion forces, occurring at the cell-surface interface. Murine fibroblasts served as a cell model for connective tissue and the strength of their adhesion to different substrate surfaces was examined by applying AFM-based single cell force spectroscopy (SCFS).

In the first part of the study (Chapter 2.1), by using femtosecond- (fs-) laser ablation two microstructures with different dimensions of surface irregularities but similar anti-wetting surface properties were generated. The attachment of fibroblasts to both microstructures as well as to polished titanium and glass surfaces was investigated by SCFS in the first 30 seconds of interaction between cells and substrate surfaces. Furthermore, the spatial organization and the geometry of surface irregularities of all surfaces were examined precisely by AFM imaging, in order to correlate the geometrical surface properties with the strength of fibroblasts adhesion. It was hypothesized that anti-wetting surfaces, produced by laser ablation, are able to inhibit the adhesion of fibroblasts.

Since increased detachment forces of fibroblasts were revealed on both produced anti-wetting microstructures in comparison to more wettable polished titanium surfaces, the hypothesis was confuted. Because the surface irregularities on both microstructures evoked cavities with nearly the same dimensions as the cell diameter, it was concluded that an increase in the cell-substrate contact area led to an enhancement of fibroblast adhesion to both microstructures in comparison to polished titanium. In addition, the adhesion of fibroblasts to glass and polished titanium surfaces, both with similar wetting properties, was significantly different. Hence, it was concluded that the topography and other properties (probably electrostatic charges) of surfaces affected the adhesion of fibroblasts more dominantly than the wetting properties of surfaces. Furthermore, the investigation of cell proliferation after three days of cultivation on different substrate surfaces revealed no changes in the
proliferation rates of fibroblasts, when they were cultured on different titanium surfaces (structured and non-structured). Significant changes were observed, if the proliferation of cells on titanium surfaces were compared to that on plastic surfaces (Petri-dish, see chapter 2.1). These results, also indicate that the proliferation of cells depends more on other surface properties than on wetting properties, since also plastic surfaces showed the same wetting properties as polished titanium.

The second part of the study was performed to examine the effects of two biocompatible and hydrophilic polymers (PDMAA and PEtOx) with protein repellent surface properties on the adhesion of fibroblasts (chapter 2.2). Therefore, the polymers were immobilized onto glass plates and the strength of fibroblasts adhesion to the produced surfaces as well as to pure glass surfaces was measured using SCFS. Furthermore, the temporal development of cell adhesion to all substrate surfaces was determined by varying the cell-surface interaction time (5, 30 and 180 seconds). In addition, the size of cells was correlated with their detachment forces in order to study the relation between both parameters. In comparison to adhesive glass surfaces, statistically significant lower detachment forces were generated as cells were retracted from PDMAA and PEtOx surfaces after all different interaction times. In addition, after 180 seconds of cell-surface interaction the adhesion of fibroblasts to both polymers was strengthened by lower factors, compared to their adhesion to control glass surfaces. Since the adhesion of cells to substrate surfaces is known to be mediated not only by integrin receptors but also by hyaluronan, it was concluded that hydrophilic surfaces of PDMAA and PEtOx led to a contemporary inhibition of both the integrin- and the hyaluronan-mediated cell adhesion. Moreover, it was shown that more adhesive cells are subjected to more mechanical deformation of the cell body as they are retracted from the adhesive glass surfaces.

The results of this study regarding the physical modification of surfaces indicate that the modification of physical surface properties should rely on other mechanisms than the anti-wetting surface properties. In this context, the shape and the size of the tips of surface irregularities as well as their spatial organization over the substrate surfaces is suggested to affect the adhesion of cells more predominantly than the wetting properties of generated surfaces. In a study performed by Arnold et al. (2004), a distance of more than 70 nm between two single integrin binding sites led to decreased adhesion, spreading and proliferation of MC-3T3 osteoblasts and murine 3T3 fibroblasts (cell-adhesive nanoparticles with a diameter of ~ 8 nm were used for
providing single integrin binding sites, more information in chapter 1.4). In another study, a distance of 50-100 nm between different integrin binding sites was shown to lead to the inhibition of cell adhesion in different stem cell types\textsuperscript{88}. Taking these results into account, surface topographies with very sharp spike-like irregularities (tip diameter ~ 10 nm) and a spike to spike distance of more than 70 nm are suggested to diminish the adhesion of different cell types by interfering the integrin clustering and thus the development of cell adhesion. Nevertheless, the dimension of cells should be considered, since if the spike to spike distances are too large, cells would sink their bodies into the cavities between different spikes and try to attach to the available areas. The exact dimensions of surface irregularities and their spatial organization over implant surfaces should be investigated and optimized in further studies.

The active electrodes of CIs are made of platinum. As described before, the adhesion of cells to substrate surfaces depends not only on surface topography and wettability, but also on the electrostatic charges of the substrate surfaces. Since the electrostatic charge of platinum surfaces is probably different than that of titanium, there is the need of investigating the adhesion of cells to platinum surfaces. Furthermore, since different cell types vary in their size and morphology it would be interesting to study the effects of microstructures on different cell types (e.g. human fibroblasts and murine fibroblasts).

The electrode carriers of CIs consist of very soft medical silicone. Soft materials for electrode carriers are desired since the implant is subjected to deformation and torsion as it is inserted into the scala tympani surgically. Furthermore and obviously, soft materials would induce less injury to the organism as they are inserted into the body. Thus, it is desired to maintain the physical properties of CI electrode carriers and to reduce the content of connective tissue capsule around them at the same time. Therefore, coating the silicone surfaces with very thin polymer monolayers (thickness ~ 10 nm\textsuperscript{107}, which would inhibit the unspecific protein adsorption and the adhesion of fibroblasts is very desirable. The results of this study indicate such properties for both PDMAA and PEtOx. Since both polymers were immobilized onto glass surfaces, the feasibility to coat silicone surfaces with these polymers should be studied. Furthermore, studies to investigate the effects of such surface coatings on the elasticity of electrode carrier materials are highly recommended.

The results of the present study prove the feasibility of atomic force microscopic SCFS for investigating the effects of physiochemical surface properties on cell
adhesion. The method gave information not only about the strength of the cell adhesion, but also about mechanical properties of cells, such as contact stiffness. Furthermore, using the method the investigation of the temporal development of cell adhesion forces was possible. In addition, the ability of AFM to investigate the surface topography precisely was used to define a new parameter for defining the spatial organization of the micro-structured surfaces, namely the mean distance between two neighbouring structures (MDNS) (Chapter 2.1)$^{106}$. Since the spatial organization of substrate surface irregularities is suggested to give more cell adhesion relevant information than the average roughness of surfaces (more information in chapter 1.7), it is recommended to characterize surface topographies by using parameters, such as MDNS for studying the correlation between cell adhesion and surface topography.
References


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Declaration

I hereby declare that I have autonomously carried out my Ph.D thesis entitled:

“Introduction to the Nano World of Cochlear Implant Surfaces”

I did not receive any assistance from in return for payment by consulting agencies or any other person. No one received any kind of payment for direct or indirect assistance in correlation to the content of the submitted thesis.

I conducted the project at the following institution:

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The thesis has not been submitted elsewhere for an exam, as thesis or for evaluation in a similar context.

I hereby affirm the above statements to be complete and true to the best of my knowledge.

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Pooyan Aliuos, August 2012