Production and physiochemical characterization of self-assembled monolayers on titanium surfaces and their influence on fibroblast behaviour

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Diploma Thesis

Production and Physiochemical Characterization of Self–Assembled Monolayers on Titanium Surfaces and their Influence on Fibroblast Behavior.

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October 2000
Abstract

The effects of surface topography and chemistry on human gingival fibroblasts behavior were studied *in-vitro*. Two different surface topographies, a smooth and a rough (SLA) titanium surface were modified by a self assembly monolayer (SAM) technique based on functionalized alkanephosphat/alkanephosphonate. Smooth and rough surfaces surfaces were modified producing hydrophobic (-CH$_3$ functionalized SAM) and hydrophilic (-OH functionalized SAM) surfaces. The rough surfaces were also modified by the SAM technique in order to obtain positively charged (-NH-(CH$_2$)-CH$_3$ functionalized SAM) and negatively charged surfaces (-OPO$_3^{2-}$ functionalized SAM).

X-ray Photoelectron Spectroscopy (XPS) and water Contact Angle measurements (CA) were used to characterize the modified surfaces. Cell morphology was investigated by Secondary Electron Microscopy (SEM) and Confocal Laser Scanning Microscopy (CLSM). Further, CLSM was used to observe cytoskeletal organization and to determine cell thickness. Cell area and perimeter were determined from the digital CLSM pictures. XPS measurements confirm the adsorption of the functionalized molecules at both the smooth and the rough surfaces. Consequently, the surfaces exhibit CA (advancing/receding) of 102°/80° for the smooth hydrophobic surfaces and 75°/61° for the smooth hydrophilic surfaces, while the topography effect of the SLA surfaces yields to a CA of 150°/148° for the hydrophobic surfaces, 108°/<10° for the hydrophilic surfaces and 132°/<10° for both the positively and negatively charged surfaces.

Fibroblasts were cultured on all surfaces in medium with 15% serum for 24 h. In the case of hydrophilic and hydrophobic surfaces, more cells attached to the smooth surface compared to SLA surface. Moreover, rough surfaces inhibit actin stress fibers formation. This observation also could be obtained on negatively and positively charged surfaces. Furthermore, cell thickness is dependent on the topography. However, cell thickness and area are also influenced by surface chemistry. Cell seeded on negatively charged surfaces are thicker but have a smaller area compared to cell seeded on positively charged surfaces. However, no clear differences were observed between cells cultured on hydrophilic and hydrophobic surfaces.
These observations indicate that fibroblasts attachment, grown and organization (actin stress fibers formation) in 15% serum condition is greatly influenced by the topography and in some extent by surface charge but not by the hydrophobicity of the surface.
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1 Introduction

Titanium, either commercially pure or in form of alloys such as Ti6Al4V or Ti6Al7Nb, is among the most commonly used implant material [1], particular for dental, orthopedic and osteosynthesis applications. Titanium is used as a biomaterial because of its optimal combination of mechanical properties, low specific weight and excellent corrosion resistance combined with no or little adverse tissue reaction. The corrosion resistance is the consequence of the immediate oxidation of titanium in air or moisture, where a stable, continuous and protective oxide film layer is produced on the metal surface [2]. This layer prevents further oxygen ions forms diffusing into the metal and continuing the metal oxidative process [3]. The natural oxide film composition and thickness depend on environmental conditions and may be nonstoichiometric, containing concentration gradients or structural defects. A value of 4 to 6 nm for the thickness is generally reported in the literature [4,5]. A direct contact is never established between the implant bulk material and the host tissue, but rather between the tissue and the oxide layer whose biocompatibility is a major determinant of implant performance [1,6]. Therefore it is important to characterize the implant surface and to consider the interfacial phenomena that occur between surface, biological molecules and cells since implant surfaces with different properties result in different responses at the cellular and, consequently, at the tissue level around the implant [7].

1.1 Influence of surface properties on the biological environment.

Topography and chemistry are among the surface characteristics that are relevant for biological reactions. A wide range of surface topographies can be obtained by different surface treatment processes such as etching, sandblasting, mechanical polishing, electropolishing or structuring by micromachining. There are many studies focusing on the influence of topography on cell responses: faster osseointegration for example is obtained on rough titanium surfaces [8,9], roughness alters osteoblast proliferation and
differentiation [10], cell alignment and locomotion can be influenced by grooved micromachined surfaces [11,12]. Besides biological responses, surface properties such as contact angle or free energy are influenced by the topography and can assume different value for the same chemical interface [13]. On the other hand, the chemical composition of the surface affects, besides the specific reactions with other elements, the wettability, the free energy and the electrical charge of the surface. Many studies show the influence of these surface properties on protein absorption [14,15,16]. However, the full protein adsorption process, which includes spatial conformation of the adsorbed molecules, motility, dissociation and reversible adsorption is a complex dynamic interaction, is poorly understood. Studies on the correlation between surface wettability and cell behavior have sometimes led to controversial results, indicating the complexity of such interactions. For example, some studies indicate an equivalent cell spreading on material with different surface energies [17], equivalent cell attachment on different surfaces preadsorbed with fibronectin [18] while others report an inhibition of cell attachment and proliferation on hydrophobic polymers [19, 20]. Electrical charged surfaces (e.g. positive charge of quaternary amines) have been shown to strongly promote cell attachment but do not influence cell area, shape, spreading or cytoskeletal organization [21].

1.2 Self-assembled monolayers (SAM)

Self-assembled monolayers (SAMs) are stable two-dimensional structures that are formed spontaneously by the adsorption of amphifunctional molecules from solution onto a solid substrate surface [22]. These molecules are characterized by a carbon chain (usually 12-18 carbon atoms) with a surface specific reactive head group at one end and a functional group at the other end. The reactive head groups react with the solid surface, while the functional groups form the liquid solid interface, determining the surface properties of the monolayer. Earlier studies of alkane phosphate on metal oxide surfaces show an ordered two-dimensional hexagonal patterns with an intermolecular spacing of ~ 0.5 nm and an average tilt angle of 30° (Fig.1.2-1). This arrangement represents the
equilibrium state involving attractive V.d.W forces between the carbon chains and the binding energy of the head group to the surface [23].

Figure 1.2-1 Self-assembled monolayer (SAM) of alkylphosphoric acid on titanium substrata.

The ability to precisely control the composition and properties of SAMs through the synthesis, together with the possibility to pattern functional groups in the plane of the monolayer, makes this class of surface one of the best approaches now available for systematic studies on protein adsorption and cell adhesion [24]. Hydrophobic, hydrophilic, positively charged or negatively charged surfaces can be produced by chemical modification of the terminal group of the SAM. Several systems producing SAM structures exist: alkanethiolates on gold [25,26], alkylsiloxane on hydroxylated surfaces (metal oxide [27], glass [28]), alkylsiloxane on silicon [28] or alkylphosphoric acid on metal oxides such as Al₂O₃ and Ta₂O₅ [29]. In other studies different SAM techniques have been used to obtain gradient surfaces [30,31], patterned surfaces [32] or mixed SAM from a solution of molecules with different functional groups [Tos paper]. Another possibility is the bioactivation of a surface by binding specific biological proteins to the SAM functional groups [28,34]. Moreover, the SAM technique allows one to chemically modify every kind of surface geometry and topography in an efficient way by simple immersion of the sample in the desired solution. The adsorbed molecules will form a monolayer in the nanometer scale at the solid-fluid interface, covering the entire surface without changing the surface structure in the biologically relevant roughness.
scales (> 50 nm). It is therefore possible to obtain the same chemical interface on etched, sandblasted and other kinds of structured surfaces as well as on smooth surfaces.

1.3 Biological aspects

Beside the physical properties of the implant such as E-modulus, yield strength or elongation, surface properties and material design, there are other critical factors determining cell and tissue responses such as the status of the bone, the surgical technique and the loading condition. Under appropriate circumstances, implants inserted into bone will establish and maintain a direct contact of implant to hard tissue [6]. Few studies suggest a direct chemical bond between the titanium oxide layer and the bone matrix [3], but generally there is no agreement on the nature of the contact between living cell and implants. Besides bone, two other tissues directly contact an osseous implant: epithelium and soft connective tissue. For oral implants, the predominant cellular components, which come in contact with an implant, are gingival fibroblasts, epithelial cells and osteoblast cells. It has been demonstrated that osteoblast cells attach and grow better on rough surfaces [10,8], whereas epithelial and fibroblast cells adhere more efficiently to smooth titanium surfaces [35]. One problem with dental implants is the risk of a decrease in bone contacting the implant and bone’s replacements by inflamed connective tissue. The dental implants used today are often designed with two different surfaces. The top part that comes in contact with the gingiva has a smooth surface, which allows formation of an epithelial tissue while the bottom part going into the bone is rough to facilitate osseointegration [6].

In order to understand the reaction of the host tissues with an implant, in vitro tests are often used for the evaluation of cell spreading, shape and motility [36]. Today it is recognized that protein adsorption is the first event in biological reactions and influences in a complex manner any further interaction between cells and surface. Cell responses, such as cell attachment, spreading and differentiation, are secondary and depend on the nature of the adsorbed layer of protein [24]. When a cell grown on a culture dish, most of their cell surface is separated from the substratum by a gap of more than 50 nm, which is
filled with components of the extracellular matrix (ECM). However in some places of the cell membrane, called focal contacts, this gap is reduced to 10-15 nm. Here the plasma membrane is attached to some components of the ECM, such as fibronectin and vitronectin, through specific proteins, such as integrins, located at the cell membrane. These transmembrane proteins are linked in the inner part of the cell to the cytoskeleton. The main components of the cytoskeleton are microtubules, intermediate filaments and microfilaments. Actin is the main constituent of the microfilaments, which are also called actin filaments or actin stress fibers. During cell adhesion, the actin forms stress fibers, which bind the cytoskeleton to the focal contact [37]. Besides their role as anchors for the cell, focal contacts can also transmit signals from the ECM to the cytoskeleton through the transmembrane integrin proteins [38]. Different stress fiber macrostructure may be related to changes in cell activity, metabolism and gene expression. Therefore the actin filaments and their conformation are of particular interest for the study of the reaction between cell and substrata.
1.4 Aim of the study

The aim of the study is to investigate how surfaces with different chemical properties and different topographies influence cell behavior. The first part, performed at the Laboratory of Science Surface and Technology, ETH Zurich, was dedicated to the manufacturing and characterization of the various surfaces. Four different chemically modified surfaces were produced using the SAM technique. SAMs were obtained from alkylphosphoric acid or alkylphosphonic acid with different functional groups, using a -CH₃ terminated group to produce a hydrophobic surface, -OH for a hydrophilic, -NH-CH₂-CH₃ for a positively charged and –OPO₃ for a negatively charged surface. Titanium with two different topographies was used as substratum: a smooth surface, obtained by titanium sputtering on glass, and a rough titanium surface, produced by sandblasting commercially pure (c.p.) titanium discs combined with a subsequent etching process (SLA). Hydrophilic and hydrophobic surfaces were produced on both smooth and SLA surfaces, positively and negatively charged surfaces on SLA surfaces. The chemical surface characterization was performed using X-ray photoelectron spectroscopy (XPS) and the wettability was determined by contact angle measurements (CA). In the second part of the study, performed at the Faculty of Dentistry, University of British Columbia, Vancouver, the influence of the different modified surfaces on the cell response was investigated using gingival fibroblast cells. Cell morphology and cell spreading were determined with confocal laser scanning microscopy (CLSM) using fluorescent membrane staining and with scanning electron microscopy (SEM), while cytoskeletal organization was observed with CLSM using fluorescent actin staining.
2 Materials and methods

2.1 Surface preparation and characterization

2.1.1 Substrata

Smooth surfaces were produced by depositing a titanium metal film on glass substrates (Ti/glass, Paul Scherrer Institut PSI, Switzerland). The thickness of the titanium layer is about 100 nm, which allows considering the sample as bulk titanium. Atomic force microscope (AFM) image shows the smooth Ti/glass surface (Fig. 2.1-1).

![AFM picture of the smooth Ti/glass surfaces](image)

Figure 2.1-1 AFM picture of the smooth Ti/glass surfaces

Commercial pure (c.p.) titanium discs were used to manufacture the blasted + etched surface (SLA). First, the titanium discs were particle blasted with alumina beads and then chemically etched in a hot solution of HCl/H_2SO_4 (Institut Straumann AG, CH-4437 Waldenburg, Switzerland). The etching process removes the alumina particles from the previous blasting process and superimposes a finer structure on the blasted surface. Previous characterizations of the SLA surface [39] have shown the effect of the two consecutive surface structuring processes. The result is a topography with two characteristic contributions, one in the range of 20-40 µm (produced by the blasting
process and the following particles removing during the etching process) and the second one in the range of 0.5-2 \( \mu \text{m} \) (from the etching process). A micrograph of the SLA surface taken by scanning electron microscopy (SEM) is shown in Fig. 2.1-2 [39].

![SEM micrograph of the SLA surface](image)

**Figure 2.1-2** SEM micrograph of the SLA surface [39]

The surface roughness of the smooth surface was determined using AFM (10 \( \mu \text{m} \) profile length), whereas for the rough surface non-contact laser profilometry (LPM) was applied over a scan length of 85 \( \mu \text{m} \) because of the insufficient scan range of the AFM cantilever in the z-direction. Results are shown in Tab. 2.1-1

**Table 2.1-1** Roughness analysis of the Ti/glass and SLA surfaces. \( R_a \) is the arithmetic average of the absolute values of all points on the profile; \( R_q \) is the root mean square of all the values of all points on the profile.

<table>
<thead>
<tr>
<th>Roughness parameter</th>
<th>Ti/glass</th>
<th>SLA</th>
</tr>
</thead>
<tbody>
<tr>
<td>( R_a )</td>
<td>2.48 nm</td>
<td>3.93 ( \mu \text{m} )</td>
</tr>
<tr>
<td>( R_q )</td>
<td>3.85 nm</td>
<td>4.69 ( \mu \text{m} )</td>
</tr>
</tbody>
</table>
2.1.2 Preparation of Self-Assembled Monolayers (SAM)

Four different molecules were used for the self-assembled monolayer (SAM) coating to obtain hydrophilic, hydrophobic, positively charged and negatively charged surfaces.

2.1.2.1 Production of hydrophilic and hydrophobic surfaces

The molecules used for the hydrophilic SAM were obtained from the salt \((\text{HO-(CH}_2\text{)}_{12}\text{-O-PO}_3\text{)}\text{)(NH}_4\text{)}\text{)}_2\) (12-(Hydroxy)dodecylphosphate, OH-DDP) dissolved in ultrapure water (18.2 MΩ cm), while the \((\text{CH}_3\text{-}(\text{CH}_2\text{)}_{11}\text{-O-PO}_3\text{)}\text{)(NH}_4\text{)}\text{)}_2\) (Dodecylphosphate, DDP) was used for the hydrophobic SAM. The water solutions were used as immersion bath for the adsorption of the SAMs. Ti/glass and SLA surfaces were first cleaned in an ultrasonic bath in 2-propanol for 5 min. The procedure was repeated a second time for another 5 min. using fresh 2-propanol solvent. Samples were then blow-dried with N\(_2\) and the remaining organic impurities on the surfaces were removed by O\(_2\) plasma cleaning for 3 min. After plasma cleaning, surfaces are highly activated and tend to react with the impurities present in the air. Therefore, the time before the immersion in the solution should be reduced to a minimum to allow a direct contact between the solvated molecules and the titanium surfaces rather than between impurities and the titanium surfaces. The SAMs were formed by immersion of the cleaned surfaces in the DDP or OH-DDP water solution for 48 h. After that, the samples were rinsed with ultrapure water, blow-dried with N\(_2\) and stored in air until the surface characterization or the cell cultures were performed.

2.1.2.2 Production of positively and negatively charged surfaces

A similar procedure was used to produce the positively and negatively charged SAMs. SLA surfaces were ultrasonic cleaned in 2-propanol for twice 8 min. with intermediate changing of the solvent. Samples were then blow-dried with N\(_2\) and UV cleaned for 30
min. A mixed water-organic solvent with water, methanol and ethanol (50ml; 5ml; 50ml) was used to solve 0.5mM of 12-(N-ethylamino)dodecylphosphonate CH$_3$-(CH$_2$)-NH-(CH$_2$)$_{12}$-PO$_3$H$_2$ for the production of the positively charged surfaces while for the negatively charged surface 0.5mM 1,12-dodecyldiphosphate H$_2$PO$_3$-O-(CH)$_{12}$-O-PO$_3$H$_2$, were solved in ultrapure water. As for the hydrophilic and hydrophobic surfaces, the wafers were quickly immersed in the SAM solution after the UV cleaning, avoiding long exposure of the activated surface to the air.

Previous studies on the structural chemistry of SAM on tantalum oxide have shown that a coordination bond is formed between the metal ions and the oxygen from the phosphoric/phosphonic group. This bond can involve two oxygen atoms from the phosphoric/phosphonic group (bidentate structure) or just one, leaving the other oxygen free to form an intermolecular hydrogen bond (monodentate) [23]. No evidence could be found in the literature that the same bond is formed on titanium oxide, but a possible structure is shown in Fig. 2.1-3. Table 2.1-2 summarizes all treated surfaces used in this study.

![Figure 2.1-3 Phosphate coordination to titanium oxide.](image)

**Figure 2.1-3** Phosphate coordination to titanium oxide.
Table 2.1-2 Summary of all treated surfaces used in this work.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Surface</th>
<th>Crosslinker group</th>
<th>Carbon chain group</th>
<th>Functional group</th>
<th>Properties</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ti/glass</td>
<td>smooth</td>
<td>-OPO$_3^{2-}$</td>
<td>-(CH$<em>2$)$</em>{12}^-$</td>
<td>-OH</td>
<td>hydrophilic</td>
</tr>
<tr>
<td>Ti/glass</td>
<td>smooth</td>
<td>-OPO$_3^{2-}$</td>
<td>-(CH$<em>2$)$</em>{11}^-$</td>
<td>-CH$_3$</td>
<td>hydrophobic</td>
</tr>
<tr>
<td>Ti</td>
<td>rough (SLA)</td>
<td>-OPO$_3^{2-}$</td>
<td>-(CH$<em>2$)$</em>{12}^-$</td>
<td>-OH</td>
<td>hydrophilic</td>
</tr>
<tr>
<td>Ti</td>
<td>rough (SLA)</td>
<td>-OPO$_3^{2-}$</td>
<td>-(CH$<em>2$)$</em>{11}^-$</td>
<td>-CH$_3$</td>
<td>hydrophobic</td>
</tr>
<tr>
<td>Ti</td>
<td>rough (SLA)</td>
<td>-PO$_3^{2-}$</td>
<td>-(CH$<em>2$)$</em>{12}^-$</td>
<td>-NH-CH$_2$-CH$_3$ (N-Et)</td>
<td>(+) charged</td>
</tr>
<tr>
<td>Ti</td>
<td>rough (SLA)</td>
<td>-OPO$_3^{2-}$</td>
<td>-(CH$<em>2$)$</em>{12}^-$</td>
<td>-OPO$_3^{2-}$</td>
<td>(-) charged</td>
</tr>
</tbody>
</table>

2.1.3 Characterization methods

2.1.3.1 Contact-Angle Measurement (sessile-drop technique)

By placing a droplet of distilled water on a surface, the static angle that forms at the three-phase contact line between the surface and the liquid-air interface is not unique. Surface roughness at the nanometer scale distorts the liquid droplet boundary and give rise to several metastable states separated by an activation energy. Varying the volume of the droplet by adding pulse of liquid from the tip of a suspended syringe increases the static contact angle without moving the three-phase contact point. After a critical liquid volume is reached, the droplet boundary moves over the surface and forms an almost constant angle, which is defined as the advancing contact angle. Its value is calculated at three different places on the surface where 10 angles for every droplet were measured.
Receding contact angles were taken in the same way but by withdrawing water from the droplets (decreasing the volume) and by measuring the angle again after an almost constant contact angle was obtained. The difference between the advancing and the receding contact angles (contact angle hysteresis) is also important for the characterization of the surface wettability in addition to the maximum achievable advancing contact angle. For example, a droplet of water will slide easier on a surface with little hysteresis than on a surface with higher advancing but lower receding contact angle [13]. A large difference between advancing and receding contact angles is the consequence of the resistance of the droplet boundary to move over the surface. That resistance prevents the water droplet from sliding even if the measured advancing angle is high and characteristic for hydrophobic surface. Therefore the surface wettability was investigated by measuring both advancing and receding water contact angles.

2.1.3.2 X-ray Photoelectron Spectroscopy

The chemical composition of the surfaces was investigated with X-ray Photoelectron Spectroscopy (XPS). This technique allows the determination of the binding energy of core electron by irradiating a sample with X-ray and measuring the kinetic energy (E_k) of the photoelectrons according to equation (1):

\[ E_k = h \cdot \nu - E_B - \phi \]  (1)

where \( h \cdot \nu \) is the energy of the exciting photon, \( E_B \) the binding energy and \( \phi \) the spectrometer work function.

The irradiating photons cause two kinds of electron emission. First, an electron can absorb the energy of the photon and leave the atomic orbital. This energy absorption is characterized by the quantum number of the orbital left by the electron and the spin-orbit splitting. For example 2p1/2 or 2p3/2 means that the photon absorption is caused by the electrons emitted from the 2p orbital (1/2 and 3/2 result from the spin-orbit splitting). In addition to the emitted electrons, other electrons, called Auger electron, may be emitted because of the relaxation of the excited ions remaining after the photoemission. In such a
process, electrons in outer shells fill the lower orbital vacancy, and a second electron is simultaneously emitted, carrying the energy excess. This three-electrons process is characterized by the three letters representing the atomic levels involved in the process and does not depend on the energy of the irradiating photons [40]. Because each element has a unique set of binding energies, XPS can be used to identify and determine the concentration (in atomic %) of elements. The binding energy in an atom is not only determined by the type of atom, it also depends on the chemical environment around the atom. These shifts in the electron-binding energy are measurable by XPS, which makes it possible to determine the chemical-bonding state of the detected elements. Although the X-ray themselves penetrate up to 1-10 µm into the material, the mean free path of photoelectrons is much smaller and limits the information depth, typically to 5-10 nm. Because all the information originates from the top-most atomic layers of the surface, XPS is one of the most useful methods for studying the chemical composition of surfaces.

In the present study, spectra were obtained on SAGE 100 system (Specs, Berlin) using a non-monochromatic Al Kα radiation at 320W (1486.6 eV), an electron take-off angle of 90° with respect to the sample surface and a pressure of 2.5×10⁻⁸ mbar. Survey scans over a binding energy of 0 to 1400 eV were taken with a constant electron detector pass energy of 50 eV. High resolution spectra were measured with a pass energy of 14 eV and used for quantitative determination of the binding energy, chemical bonding and atomic concentration. The total time of exposure to X-ray was usually less than 1 h. The quantitative evaluation of the resolution spectra was carried out using SpecsLab software. To convert peak area to surface concentration, Scofield sensitivity factors [41] were used.
2.1.3.3 Thermal degradation resistance of the Self-Assembled Monolayers

In the context of evaluating different sterilization procedure, the resistance to thermal degradation of the SAM structure was studied. Smooth samples with both -OH and -CH$_3$ terminated groups were put in an oven at low vacuum (~50 mbar) for 1 h, starting at 40°C up to the desired temperature with a maximum rate of 2°C/min. The chosen temperatures were 75°C, 100°C, 125°C and 150°C, which are below the decomposition temperature of polymeric carbon chains. After 1 hour the samples were taken out of the oven and let cool down in air. XPS and CA measurements were taken within the same day of the heat treatment.
2.2 Biological investigations

2.2.1 Cell culture

Fibroblast between the 8th and 12th subculture, isolated from human gingival explants were cultured in culture medium containing α-minimal essential medium (MEM Stemcell, Vancouver B.C, Canada) supplemented with 15% serum fetal clone III (HyClone, Logan UT, USA) and antibiotics at 37°C in a humidified atmosphere with 5% CO₂. Antibiotics mixture contains 100 µg/ml penicillin G (Sigma, St. Louis MO, USA), 50 µg/ml gentamicin (Sigma) and 3 µg/ml amphotericin B (Fungizone, Gibco, Grand Island, NY, USA). Upon confluence, the following procedure was used to remove the cells before seeding on the different surfaces:

1. The medium was removed from the 250 ml culture flask and washed away by rinsing in 5 ml trypsin solution (0.25% trypsin (Gibco), 0.1% glucose, citrate saline buffer, pH=7.8).
2. Another 5 ml of trypsin were added and the flask was incubated for 10-15 min at 37°C.
3. The cells were removed by pipetting up and down the liquid from the flask.
4. The cells suspension was put in a 10 ml sterile vial and 5 ml of culture medium were added to stop the action of the trypsin.
5. The vial was spun at 1500 rpm for 5 min.
6. The supernatant was gently discarded, leaving the cells at the bottom of the vial. After that, cells were resuspended by adding 10 ml of culture medium and by pipetting the liquid up and down several times.

Cell concentrations were electronically measured using a cell counter (Coulter Z1), diluting 0.5 ml of the cells suspension in 9.5 isotonic solution. The desired concentration was adjusted by adding medium.
2.2.2 Experimental conditions

Different observation methods, cell concentrations and experimental conditions were applied to study cell morphology, cell thickness and cytoskeletal organization. In the first experiment, cells were seeded on the different materials with a high cell concentration \(1.3 \times 10^5\) cells/ml. Scanning electron microscope (SEM) pictures were taken for studies of cell morphology.

Confocal laser scanning microscopy (CLSM) was used as a second method for the study of cell morphology. A lower cell concentration \((1.3 \times 10^4\) cells/ml) was chosen in order to avoid confluence of the cells and allowing the determination of the single cell morphology. For cell thickness determination cells were measured using fluorescent membrane staining. Cell thickness was partially measured directly using CLSM and partially by taking digital images for later interpretation.

Cell area and perimeter were determined from the digital CLSM pictures using the Scion Image Program (version Beta 4.0.2).

For the investigation of the serum influence, the culture medium composition was changed in order to obtain only 0, 2 and 4% serum content. Cells were seeded on hydrophilic and hydrophobic smooth surfaces with a concentration of \(5.0 \times 10^4\) cells/ml. Cytoskeletal organization was observed with CLSM using fluorescent actin staining. Cells were seeded on all the different surfaces at a concentration of \(1.3 \times 10^4\) cells/ml.

For all experiments, cells were seeded at the chosen concentrations on the different surfaces in 12-well culture plates and incubated at 37°C and 5% CO\(_2\) for 24 h.

Table 2.2-1 shows the experimental conditions and the observation methods used for the different cell studies.
### Table 2.2-1 Experimental conditions

<table>
<thead>
<tr>
<th>Determination purpose</th>
<th>Observation method</th>
<th>Cell concentration</th>
<th>Samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Morphology</td>
<td>SEM</td>
<td>(1.3 \cdot 10^5) cells/ml</td>
<td>all</td>
</tr>
<tr>
<td>Cell thickness</td>
<td>CLSM, membrane staining</td>
<td>(1.3 \cdot 10^4) cells/ml</td>
<td>all</td>
</tr>
<tr>
<td>Shape factor</td>
<td>CLSM, membrane staining</td>
<td>(1.3 \cdot 10^4) cells/ml</td>
<td>all</td>
</tr>
<tr>
<td>Serum influence, shape factor</td>
<td>SEM</td>
<td>(5.0 \cdot 10^4) cells/ml</td>
<td>smooth hydrophilic/hydrophobic</td>
</tr>
<tr>
<td>Cytoskeletal organization</td>
<td>CLSM, actin staining</td>
<td>(1.3 \cdot 10^4) cells/ml</td>
<td>all</td>
</tr>
</tbody>
</table>

#### 2.2.3 Observation methods

2.2.3.1 Scanning electron microscopy (SEM)

Scanning electron microscopy (Cambridge 260, operated at an accelerating voltage of 4-11 kV in a sample chamber vacuum between \(1 \cdot 10^{-5}\) and \(1 \cdot 10^{-6}\) Torr) was used to investigate the morphology of fibroblasts on the different SAM treated surfaces (see section 2.1.2). Because SEM requires a high vacuum and a conductive sample, cells had to be fixed, dried and coated with a thin gold layer. Digital pictures were taken with a PC equipped with a capture card and were used for morphological evaluation.
Cell preparation for SEM

According to the standard procedure used in the laboratory, the following preparation was carried out before the cell observation was carried out:

1. The culture medium was removed and the samples were quickly rinsed in wash medium (no serum or antibiotics were added).
2. Cells were fixed with a solution of 2.5% glutaraldehyde in 0.1 M phosphate buffer saline (PBS) for 1h at 4°C.
3. Samples were rinsed three times in 0.1 M PBS.

For the second stage of the preparation, two different procedures were used. The hydrophilic and the hydrophobic samples were fixed in a conventional way, while for the positively and the negatively charged samples a microwave procedure was applied [42]. It involves the use of the same chemicals, but by exploiting the atomic excitation caused by the microwave irradiation (Pelco 3470 Hornet, Redding, CA, USA), the time for each step was remarkably reduced. Table 2.2-2 summarizes the different steps for the two different procedures.

Table 2.2-2 Two different procedures (conventional and microwave) used for the cell preparation before SEM observation.

<table>
<thead>
<tr>
<th>step</th>
<th>-OH and –CH₃ , smooth and SLA surfaces</th>
<th>-N-Et and –OPO₃ , SLA surfaces</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Cells were fixed with a 1% osmium tetraoxide (OsO₄) solution in 0.1 PBS</td>
<td>30 min. at 4°C.</td>
<td>40 sec.</td>
</tr>
<tr>
<td>2. Samples were rinsed two times in PBS 0.1 M</td>
<td>2 x 5 min. at 4°C.</td>
<td>2 x 30 sec.</td>
</tr>
<tr>
<td>3. Samples were dipped in 2% tannic acid solution</td>
<td>20 min. at 4°C.</td>
<td>40 sec.</td>
</tr>
</tbody>
</table>
4. Samples were rinsed two times in PBS 0.1 M  
   2 x 5 min. at 4°C.  
   2 x 30 sec.

5. 1% osmium tetraoxide (OsO₄) in 0.1 PBS was used for the second time  
   30 min. at 4°C.  
   40 sec.

6. Samples were rinsed two times in PBS 0.1 M  
   2 x 5 min. at 4°C.  
   2 x 30 sec.

7. Cells were dehydrated in 30%  
   5 min. at 4°C  
   2 x 30 sec.

<table>
<thead>
<tr>
<th>Alcohol Solution</th>
<th>Time</th>
</tr>
</thead>
</table>
| 50%              | 5 min. at 4°C  
                  | 2 x 30 sec.  |
| 70%              | 5 min. at 4°C  
                  | 2 x 30 sec.  |
| 80%              | 5 min. at 4°C  
                  | -           |
| 90%              | 2 x 5 min. at RT  
                  | 2 x 30 sec.  |
| 95%              | 2 x 5 min. at RT  
                  | -           |
| 100%             | 2 x 5 min. at RT  
                  | 3 x 40 sec.  |

The glutaraldehyde solution covalently cross-links the protein molecules to their neighbors while the osmium solution stabilizes the proteins and the lipid bilayer by oxidizing and cross-linking the C=C double bond. Its action is increased by using a 2% tannic acid solution in the middle of the fixing stage. After dehydration, all water in the cells is exchanged for alcohol, which can be removed by the critical point drying process (CPD, Ladd, Burlington VT, USA). Thereby, samples were put in a small chamber at 4°C. The chamber was filled 4-5 times with liquid CO₂ at a pressure of ~700 psi to replace the alcohol in the cells. Then the CO₂ is heated up to 39 °C and 1300 psi, where it becomes gas without a phase transition but over the gas-liquid critical point. That allows the liquid CO₂ to become gas without surface tension resulting in a minimal risk of shrinking. Finally, the sample were coated with a ~20 nm gold layer (10mA sputtering current, 50-80 mTorr vacuum, 2 min.).
Confocal laser scanning microscopy (CLSM) is a relatively new light microscopical imaging technique, which has found wide applications in biological sciences. The primary value of the CLSM is its ability to produce optical sections through a 3-dimensional specimen, for example an entire cell or a piece of tissue. Each optical section contains, to a good approximation, information from only one focal plane. One of the most important imaging modes in biological laser scanning confocal microscopy is fluorescence, which involves the use of antibodies labeled with fluorophore to detect substances within a specimen. The incident radiation excites the fluorophore, which emits light that can be used to visualize specific part of the tissue where the antibodies have attached. In Fig.2.2-1, the confocal principle is illustrated schematically. To image the specimen point by point, a laser beam is deflected stepwise in the x- and y-direction by a scanning unit before it is reflected by a dichroic mirror (beam splitter) so as to pass through the objective lens of the microscope, and is focused onto the specimen. The emitted, longer-wavelength fluorescent light is collected by the objective lens passes through the dichroic mirror (transparent for the longer wavelength) and is focused into a small pinhole (the confocal aperture) to eliminate all the out-of-focus light. All light coming from regions of the specimen above or below the plane of focus will therefore not contribute to the recorded image. By using a computer controlled CLSM, section at different depth can be stored and the digital images can be processed in a 3-D reconstruction of the specimen.
In this study, a CLSM (Zeiss LSM 100) was used for the visualization of membrane and actin. The membrane was stained with the fluorescent aldehyde-reactive probe fluorescein-5-thiosemicarbazide (FITC). The cell-surface glycoconjugates were oxidized and formed aldehyde groups that reacted with the staining substance. The membrane staining was used for visualizing cell morphology and for the determination of the cell thickness by measuring the height difference between the two focus planes at the top and at the bottom of the cell. For the SLA surfaces, the cell height was measured at the nucleus. In order to visualize the cytoskeletal organization, the actin stress fibers were stained with fluorescence staining, which involves the use of an actin binding protein (Phallotoxin) conjugated with a fluorophore (Rhodamine). Tab 2.2-3 shows the staining substances used for visualizing the membrane and the actin stress fiber, the adsorbed and the emitted light. A laser light with the corresponding wavelength is used to excite the fluorophores while a filter is used to select only the light emitted from the stained part of the cell.
### Table 2.2-3 Wavelength of the exciting and emitted light from the fluorophores

<table>
<thead>
<tr>
<th>Stained element</th>
<th>Fluorescent staining</th>
<th>Exciting light</th>
<th>Emitted light</th>
</tr>
</thead>
<tbody>
<tr>
<td>Membrane</td>
<td>FITC</td>
<td>490 nm</td>
<td>525 nm</td>
</tr>
<tr>
<td>Actin stress fibers</td>
<td>Rhodamine</td>
<td>540 nm</td>
<td>580 nm</td>
</tr>
</tbody>
</table>

Images of the membrane were used for the determination of the cell area and perimeter while images of the actin stress fiber were used for the discussion of the surface influence in the cytoskeletal organization. A cell shape factor $\phi$ representing the spreading extension was also calculated. It is defined as:

$$
\phi = \frac{4 \cdot \pi \cdot A}{p^2}
$$

where $A$ is the area of the cell and $p$ is the perimeter. A round cell yields to a shape factor of 1, while increased spreading results in an increased $\phi$.

#### Cell preparation for CLSM

The following procedure protocol was used for the cell fixation and for the double staining of the membrane and the actin stress fibers.

1. The culture medium was removed from the flask, the cells were quickly rinsed in wash medium and then fixed with a 3.7% formaldehyde solution in 0.1M PBS for 10 min at RT.
2. Samples were subsequently washed three times with cytoskeletal stabilizing (CS) Buffer (Opas, 1989).
3. Cells were oxidized in 4.2mM NaIO$_4$ for 30 min. at 4°C.
4. Samples were well rinsed in 0.1 M PBS.
5. Membrane was stained with a 10mM fluorescein IsoThiosemicarbazide (FITC, Molecular Probes Inc.) for 30 min. at 37°C. Thereby, a droplet (~40 µl) of this solution was put on a dry 12-well plate for every sample. The samples were taken out of the PBS, dried by placing the edge of the sample against an absorbent surface (e.g. tissue) and gently placed onto the droplet of stain. In order to maintain a humid environment, one of the dishes in the 12-well plates was filled with water. The 12-well plate was then closed and protected against light.

6. Samples were well rinsed 3 times in 0.1 M PBS.

7. Nonspecific protein binding sites were blocked with 1% bovine serum albumin (BSA, Sigma) in PBS for 30 min. at 37°C.

8. Actin stress fibers were stained using phallotoxin-rhodamine conjugate (Molecular Probes, Inc., Eugene, USA) for 30 min at RT. Phallotoxin was obtained from the ethanol solution where it was dissolved by first evaporating the alcohol. A volume of 1% BSA in 0.1 M PBS, which was twice the original alcohol-phallotoxin solution, was added in order to dissolve the dried phallotoxin. The samples were stained using the same procedure as for the membrane staining.

9. The samples were finally well rinsed in 0.1 M PBS and stored at 4°C protected from light upon use.
3 Results and discussion

3.1 Surface characterization

3.1.1 Contact angle

The measured advancing and receding contact angles of the samples used for the biological investigations are given in Tab. 3.1-1.

**Table 3.1-1 Contact angles of the samples**

<table>
<thead>
<tr>
<th>Surface</th>
<th>Functional group</th>
<th>Topography</th>
<th>Water contact angle</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>advancing [°] receding [°]</td>
</tr>
<tr>
<td>Ti/glass</td>
<td>-CH$_3$</td>
<td>smooth</td>
<td>102±3 80±7</td>
</tr>
<tr>
<td>Ti/glass</td>
<td>-OH</td>
<td>smooth</td>
<td>75±5   61±5</td>
</tr>
<tr>
<td>SLA</td>
<td>-CH$_3$</td>
<td>rough</td>
<td>150±2 148±2</td>
</tr>
<tr>
<td>SLA</td>
<td>-OH</td>
<td>rough</td>
<td>108±2  &lt; 10</td>
</tr>
<tr>
<td>SLA</td>
<td>-NH-CH$_2$-CH$_3$</td>
<td>rough</td>
<td>132±1  &lt; 10</td>
</tr>
<tr>
<td>SLA</td>
<td>-OPO$_3^{2-}$</td>
<td>rough</td>
<td>133±3  &lt; 10</td>
</tr>
</tbody>
</table>

The -CH$_3$ terminated SAM surfaces show higher advancing and receding CA as all other surfaces, as expected for hydrophobic samples. The relative small difference between the advancing CA of the hydrophilic and the advancing CA of hydrophobic smooth surfaces could suggests that the SAM was not perfectly formed on the samples. However measurements of the advancing and receding CA on rough surfaces clearly show the different wettability properties, especially when the receding CA is considered. In the case of hydrophilic and hydrophobic modification, the advancing CA is considerably increased on rough surfaces. In addition, all rough surfaces except the hydrophobic ones show extremely low receding CA and large hystereses. For the interpretation of the
results on rough surfaces, three main phenomena have to be considered [43]. First, the
three-phase contact line is highly irregular (contorted). This contortion does not represent
an ideal condition and the measured advancing and receding CA may not be correlated to
the surface free energy of the system. Moreover, the three-phase contact line might be
different in the case of the advancing and receding CA. A second point to take in
consideration is the existence of mechanical and free energy barriers induced by
topographical features between the metastable states. This may prevent the transition to
the more energetically favorable (equilibrium) droplet configurations. Additionally, for
the hydrophobic rough surfaces, it is well known that water does not penetrate between
asperities. As a consequence, the water is in contact with both surface irregularity and air
cavities. This incomplete wetting, which is known to increase the water CA, must also be
considered.

In summary, it is very difficult to measure the “true” (equilibrium) advancing and
receding CA on “real” surfaces efficiently. However, it can be concluded from the CA
measurements that the -CH₃ modified surface repels water while the –OH modified are
easily wetted, especially for the receding angle / rough surface case.

3.1.2 X-ray Photoelectron Spectroscopy

All XPS survey spectra are dominated by the O, Ti, C and P emission peak intensities. Ti
originates from the metallic substrata and oxide layer, O from the titanium oxide layer,
from the crosslinker group and in case of hydrophilic and negatively charged surfaces
from the functional group of the SAM. C derives from the SAM carbon chain and from
possible surface contaminations. For the SAM with the N-Et functional group (positively
charged surface) a small amount of N was also observed. Fig 3.1-1 shows a typical XPS
survey spectrum of the –CH₃ terminated SAM treated surface used in this study
Figure 3.1-1 Typical XPS survey spectrum of a –CH₃ terminated SAM modified Titanium surface.

High resolution detail spectra taken with smaller pass energy were used for the elemental quantification and the determination of the chemical environments of the atoms present at the surfaces. All spectra were referenced to the aliphatic hydrocarbon C1s signal at 285.0 eV. The chemical composition (atom-%) of the different surfaces is presented in Tab 3.1-2. These values are calculated assuming a homogeneous composition of the surface, which is clearly not the case in case of an ordered SAM. Therefore the values should only be used for comparative purpose.

<table>
<thead>
<tr>
<th>Substrata</th>
<th>Functional group</th>
<th>Ti</th>
<th>O</th>
<th>C</th>
<th>P</th>
<th>N</th>
<th>Ratio C/Ti</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ti/glass</td>
<td>Ref</td>
<td>18.2</td>
<td>73.5</td>
<td>8.3</td>
<td>-</td>
<td>-</td>
<td>0.46</td>
</tr>
<tr>
<td>Ti/glass</td>
<td>-CH₃</td>
<td>19.8</td>
<td>46.6</td>
<td>30.4</td>
<td>3.2</td>
<td>-</td>
<td>1.54</td>
</tr>
<tr>
<td>Ti/glass</td>
<td>-OH</td>
<td>17.8</td>
<td>48.1</td>
<td>30.6</td>
<td>3.4</td>
<td>-</td>
<td>1.72</td>
</tr>
<tr>
<td>SLA</td>
<td>Ref</td>
<td>27.4</td>
<td>59.0</td>
<td>13.5</td>
<td>-</td>
<td>-</td>
<td>0.49</td>
</tr>
<tr>
<td>SLA</td>
<td>-CH₃</td>
<td>15.8</td>
<td>41.0</td>
<td>39.5</td>
<td>3.7</td>
<td>-</td>
<td>2.50</td>
</tr>
<tr>
<td>SLA</td>
<td>-OH</td>
<td>15.1</td>
<td>42.8</td>
<td>37.1</td>
<td>5.1</td>
<td>-</td>
<td>2.46</td>
</tr>
<tr>
<td>SLA</td>
<td>-N-Et</td>
<td>18.7</td>
<td>44.0</td>
<td>32.4</td>
<td>3.1</td>
<td>1.9</td>
<td>1.73</td>
</tr>
<tr>
<td>SLA</td>
<td>-PO₄²⁻</td>
<td>14.6</td>
<td>42.5</td>
<td>36.3</td>
<td>6.6</td>
<td>-</td>
<td>2.49</td>
</tr>
</tbody>
</table>
Significantly more carbon was found on the SAM modified surfaces than on the reference surfaces, confirming the presence of the SAM molecules. Higher carbon and lower Ti substrata intensities were found on the SAM covered SLA surfaces. This is expected since for 90° electron take-off angle the effective locale take-off angle is smaller than 90°, resulting in an higher surface sensitivity (higher C/Ti ratios) in comparison to the smooth surface case. As expected, P and N were only detected on the corresponding modified surfaces. A larger amount of P was found for the –OPO$_3$ samples, confirming the presence of biphospate molecules at the surface.

As already discussed in section 2.1.3.2, XPS also gives information about the chemical bonds. Therefore in the next paragraph the high resolution spectra of O1s and C1s of the hydrophilic and hydrophobic samples (Fig. 3.1-2 and Fig. 3.1-3) will be discussed in more detail.

The O1s peak was fitted with three contributions for the hydrophobic and four contributions for the hydrophilic SAM, according to the different bond energies for the O atom in the oxide layer and in the SAM molecules. The C1s peak was fitted with two contributions corresponding to the two types of carbons (C-C, C-O) in the SAM molecules. Energies and bond assignment are shown in Table 3.1-3. Curve fitting of the C1s and O1s signal was performed using Gaussian-Lorentzian curves with a branching ratio of 0.50 for the C1s(1) and O1s(1), 0.70 for the C1s(2) and 0.90 for the O1s(2), O1s(3) and O1s(4) peaks.
**Figure 3.1-2** Detail C1s spectra of the smooth –CH₃ (a), SLA –CH₃ (b), smooth –OH (c) and SLA –CH₃ (d) modified surfaces.

**Figure 3.1-3** Detail O1s spectra of the smooth –CH₃ (a), SLA –CH₃ (b), smooth –OH (c) and SLA –CH₃ (d) modified surfaces.
Table 3.1-3 XPS binding energies (BE), half-maximum height (fwhm), peak area and area% for -CH$_3$ and -OH modified samples.

<table>
<thead>
<tr>
<th>peak assignment</th>
<th>CH2, CH3</th>
<th>C-O-P</th>
<th>C-O-H</th>
<th>Ti-O</th>
<th>P=O</th>
<th>P-O-H</th>
<th>C-O-H</th>
</tr>
</thead>
<tbody>
<tr>
<td>surface</td>
<td>C1s(1)</td>
<td>C1s(2)</td>
<td>O1s(1)</td>
<td>O1s(2)</td>
<td>O1s(3)</td>
<td>O1s(4)</td>
<td></td>
</tr>
<tr>
<td>Ti/glass CH3</td>
<td>BE (eV)</td>
<td>285.0</td>
<td>286.8</td>
<td>530.2</td>
<td>531.5</td>
<td>532.8</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>fwhm</td>
<td>1.55</td>
<td>1.75</td>
<td>1.55</td>
<td>1.70</td>
<td>1.70</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>area</td>
<td>6494</td>
<td>985</td>
<td>22504</td>
<td>8219</td>
<td>3141</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>area %</td>
<td>86.8</td>
<td>13.2</td>
<td>24.3</td>
<td>9.3</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Ti/glass OH</td>
<td>BE (eV)</td>
<td>285.0</td>
<td>286.6</td>
<td>530.0</td>
<td>531.3</td>
<td>532.6</td>
<td>533.4</td>
</tr>
<tr>
<td></td>
<td>fwhm</td>
<td>1.55</td>
<td>1.75</td>
<td>1.55</td>
<td>1.70</td>
<td>1.70</td>
<td>1.70</td>
</tr>
<tr>
<td></td>
<td>area</td>
<td>5670</td>
<td>1770</td>
<td>21481</td>
<td>7816</td>
<td>3663</td>
<td>1589</td>
</tr>
<tr>
<td></td>
<td>area %</td>
<td>76.2</td>
<td>23.8</td>
<td>22.6</td>
<td>10.6</td>
<td>4.6</td>
<td>-</td>
</tr>
<tr>
<td>SLA CH3</td>
<td>BE (eV)</td>
<td>285.0</td>
<td>286.7</td>
<td>530.3</td>
<td>531.5</td>
<td>532.8</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>fwhm</td>
<td>1.55</td>
<td>1.75</td>
<td>1.55</td>
<td>1.70</td>
<td>1.70</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>area</td>
<td>5048</td>
<td>804</td>
<td>11292</td>
<td>4378</td>
<td>1951</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>area %</td>
<td>86.3</td>
<td>13.7</td>
<td>24.8</td>
<td>11.1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>SLA OH</td>
<td>BE (eV)</td>
<td>285.0</td>
<td>286.5</td>
<td>530.0</td>
<td>531.3</td>
<td>532.6</td>
<td>533.4</td>
</tr>
<tr>
<td></td>
<td>fwhm</td>
<td>1.55</td>
<td>1.75</td>
<td>1.55</td>
<td>1.70</td>
<td>1.70</td>
<td>1.70</td>
</tr>
<tr>
<td></td>
<td>area</td>
<td>4375</td>
<td>1391</td>
<td>11406</td>
<td>4265</td>
<td>2564</td>
<td>1092</td>
</tr>
<tr>
<td></td>
<td>area %</td>
<td>75.9</td>
<td>24.1</td>
<td>59.0</td>
<td>22.1</td>
<td>13.3</td>
<td>5.7</td>
</tr>
</tbody>
</table>

The binding energies of the corresponding peaks obtained by fitting are almost constant for all the samples. A small but consistent difference of ~0.2 eV was found between the peaks of the -CH$_3$ samples and the -OH samples, except for the reference C1s(1) peak. Besides the aliphatic hydrocarbon peak (C1s(1)), a second C1s contribution (C1s(2)) was found at 286.5-286.8 eV, which is associated with C-O-P and C-O-H bond states [23]. In the case of oxygen, the main O1s peak (O1s(1)) at 530.0-530.2 eV was related to Ti-O (from the Ti oxide layer). The second O1s peak (O1s(2)) at 531.3-531.5 eV was assigned to the P=O and to the O coordination to Ti, the peak O1s(3) at 532.6-532.8 to the P-O-H and P-O-C while the peak O1s(4) at 533.4 eV was associated with the C-O-H bond.

More information about the different C1s and O1s contributions can be obtained by calculating the following ratios from the concentrations (at%) presented in Table 3.1-3: C1s(1)/C1s(2), O1s(1)/Ti2p and (O1s(2)+O1s(3))/P2p. The results are summarized in Table 3.1-4 and compared with the expected theoretical ratios. The theoretical ratio between the two C1s contributions was calculated from the stoichiometry and is 11/1 = 11 for the –CH$_3$ sample (DDP) and 10/2 = 5 for the –OH samples (OH-DDP).
measured ratio was found to be lower than the theoretical (6.59 and 6.28 for the DDP, 3.20 and 3.15 for the OH-DDP). Because the C1s peak was fitted with two contributions (C-C and C-O), the binding energy of the two carbon bonds C-O-H and the C-O-P were fitted with a single contribution but with a large half-maximum height (fwhm 1.75). This could have lead to an overestimation of the C1s(1) peak to the detriment of the C1s(2) peak. Hence, the measured ratio is different from the expected one calculated the stoichiometry. However, in the case of the –CH3 samples, the calculated ratio was twice. That ratio corresponds to the molecular structure of the molecules, which consists of two C-O bonds for the OH-DDP and just one for the DDP.

Assuming that just the two types of coordination as shown in Fig. 2.1-3 are present, the theoretical ratio (O1s(2)+O1s(3))/P2p is 4 for every molecule, independent of the coordination structure ( (3+1)/1 = 4 for the monodentate, (2+2)/1=4 for the bidentate). The calculated ratio were found to be slightly higher for all samples (4.94, 4.53, 4.48) except for the -OH SLA (3.25). In this case, the high measured P content (5.1 at%) could be the reason for the lower ratio. However, these results are close enough to confirm the theoretical ratio of 4. The intensity distribution between the O1s(1), O1s(2) and O1s(3) contributions can be verify by the calculation of the O1s(1)/Ti2p ratio. The O1s(1) signal originates from the TiO2 oxygen and the ratio O1s(1)/Ti2p should therefore theoretically be 2. For all samples, a slightly lower ratio compared to the theoretical one was found. This indicates that by the fit of the O1s spectra, the O1s(1) contribution was probably underestimated to the detriment of O1s(2) and/or O1s(3).

Table 3.1-4 XPS atomic percent for Ti, P and for the individual C1s(1), C1s(2), O1s(1), O1s(2) and O1s(3) peaks calculated for hydrophobic and hydrophilic samples. Theoretical values are calculated from stoichiometry.

<table>
<thead>
<tr>
<th>surface</th>
<th>Ti [at%]</th>
<th>P [at%]</th>
<th>C(1) [at%]</th>
<th>C(2) [at%]</th>
<th>O(1) [at%]</th>
<th>O(2) [at%]</th>
<th>O(3) [at%]</th>
<th>at%O(2)+at%O(3)</th>
<th>at%P</th>
<th>at%O(1)</th>
<th>at%C(1)</th>
<th>at%C(2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ti/glass CH3</td>
<td>19.8</td>
<td>3.2</td>
<td>86.8</td>
<td>13.2</td>
<td>31.0</td>
<td>11.3</td>
<td>4.3</td>
<td>4.94</td>
<td>4</td>
<td>1.56</td>
<td>2</td>
<td>6.59</td>
</tr>
<tr>
<td>Ti/glass OH</td>
<td>17.8</td>
<td>3.4</td>
<td>76.2</td>
<td>23.8</td>
<td>29.0</td>
<td>10.5</td>
<td>4.9</td>
<td>4.53</td>
<td>4</td>
<td>1.63</td>
<td>2</td>
<td>3.20</td>
</tr>
<tr>
<td>SLA CH3</td>
<td>15.8</td>
<td>3.7</td>
<td>86.3</td>
<td>13.7</td>
<td>29.9</td>
<td>11.6</td>
<td>5.2</td>
<td>4.48</td>
<td>4</td>
<td>1.89</td>
<td>2</td>
<td>6.28</td>
</tr>
<tr>
<td>SLA OH</td>
<td>15.1</td>
<td>5.1</td>
<td>75.9</td>
<td>24.1</td>
<td>27.5</td>
<td>10.3</td>
<td>6.2</td>
<td>3.25</td>
<td>4</td>
<td>1.83</td>
<td>2</td>
<td>3.15</td>
</tr>
</tbody>
</table>
In summary, XPS data confirm the presence of the SAM molecules on the surface. By the SAM modification of the titanium samples, surfaces with different chemical compositions were formed.

3.1.3 Thermal degradation resistance

The contact angles of the smooth -CH$_3$ and -OH modified samples after thermal treatment (see section 2.1.3.3) are presented in Fig. 3.1-4 and Fig. 3.1-5. Both advancing and receding CA are almost constant over the investigated temperature range and are comparable to the untreated surfaces which were not exposed to thermal treatment. From XPS measurements, the ratio between the atomic concentration of the elements C and Ti were calculated to detect possible loss of the SAM, while the ratio between the atomic concentration of C and O were calculated in order to investigate the stability of the functional group. The results are presented in Fig. 3.1-6 and Fig. 3.1-7. XPS investigations confirm the stability of the SAM over the investigated temperature range.

![Graph](image)

**Figure 3.1-4** Advancing and receding contact angles for the SAM with -CH$_3$ functional group after a heat treatment for 1 hour at different temperatures (n=30 for each surface; mean value ± standard deviation).
Figure 3.1-5 Advancing and receding contact angles for the SAM with -CH3 and for SAM with -OH functional group after a heat treatment for 1 hour at different temperatures (n=30 for each surface; mean value ± standard deviation).

Figure 3.1-6 C/Ti atomic ratio for SAM with -CH3 and for SAM with -OH functional group after a heat treatment for 1 hour at different temperatures. Ratios were determined by XPS measurement.
Figure 3.1-7 C/O atomic ratio for SAM with -CH₃ and -OH functional after a heat treatment for 1 hour at different temperatures. Ratios were determined by XPS measurement.
3.2 Biological investigations

3.2.1 Cell morphology

3.2.1.1 Hydrophobic and hydrophilic surfaces

Overview SEM micrographs of the hydrophobic smooth and SLA surfaces are presented in Fig. 3.2-1. On the smooth surface, the cells were almost confluent after 24 h incubation cultured with an initial cell concentration in the culture medium of $1.3 \times 10^5$ cells/ml. A clear observation of an individual cell was not possible even by a higher magnification as shown in Fig. 3.2-2. In contrast, the number of cells attached on the SLA surface after the same period of incubation and the same initial cell concentration was much smaller. Single cells can be observed as shown in Fig. 3.2-3, where a fibroblast cell forms a bridge over a surface pit. This cell behavior was observed consistently in all areas sampled on the surface.
Figure 3.2-1 Overview SEM micrograph of fibroblasts cultured on hydrophobic smooth surface (a) and SLA surface (b)
**Figure 3.2-2** SEM micrograph of fibroblasts cultured on smooth hydrophobic surface

**Figure 3.2-3** SEM micrograph of fibroblasts cultured on SLA hydrophobic surface
The same result was observed on smooth and SLA hydrophilic surfaces. More cells attached to the smooth surface compared to the SLA surface. However, no evident morphological differences were observed between the hydrophobic and the hydrophilic surfaces on either smooth or SLA topography. Fig. 3.2-4 shows an example of fibroblast on hydrophobic and hydrophilic SLA surfaces.

Figure 3.2-4 SEM micrograph of fibroblasts cultured on (a) hydrophobic and (b) hydrophilic SLA surface
Serum proteins are known to play an essential role in the first steps of cell attachment. In order to investigate the influence of serum, fibroblasts were cultured on hydrophobic and hydrophilic smooth surfaces with variable serum content of 0, 2 and 4% in the medium. Fig. 3.2-5 shows a SEM micrograph of fibroblasts cultured with 0% serum on the two surfaces. As expected, more round cells can be observed on hydrophobic than on hydrophilic surfaces. It can be concluded, that in absence of serum, cells spread more on the hydrophilic than on the hydrophobic surfaces.

**Figure 3.2-5** Fibroblasts cultured on (a) hydrophobic and (b) hydrophilic smooth surface after 24 h incubation in absence of serum.
With increasing serum content the differences become smaller. With 4% serum in the culture medium, similar cell morphology was found on both hydrophobic and hydrophilic surfaces, with more unspread cells on the hydrophobic surface as shown in Fig. 3.2-6.

Figure 3.2-6 Fibroblasts on (a) hydrophobic and (b) hydrophilic smooth surface after 24 h incubation with 4% serum.
This tendency of serum to make hydrophobic and hydrophilic surfaces appear similar to cells is probably maintained with an increasing serum content. Therefore similar cell morphologies were observed with normal serum content (15%).

Investigation of the fibroblast behavior on hydrophobic and hydrophilic smooth and SLA surfaces was also performed using confocal scanning laser microscopy (CLSM). The advantage of CLSM is that it does not require high vacuum conditions and cells can be observed in liquid medium. Therefore the preparation for CLSM investigation is gentle compared to the SEM preparation: cell shape is less affected and CLSM pictures are more accurate for cell area and perimeter measurements on smooth surface. Ten pictures per hydrophobic or hydrophilic surface were taken and used for the measurement of area and perimeter. Fig. 3.2-7 shows a typical CLSM picture of fibroblasts on a hydrophilic smooth surface.

![CLSM image of fibroblast cell on hydrophilic smooth surface.](image)

**Figure 3.2-7** CLSM image of fibroblast cell on hydrophilic smooth surface.

Thereby cells were focused on the plane with maximal cell extension, in order to obtain a projection of the cells in the vertical direction respect to the sample surface.
Determination of cells morphology seeded on the SLA surfaces is more difficult to determine because on a rough surface the fibroblasts maximal extension does not lie on a horizontal plane and many pictures taken at different focus planes are needed in order to obtain information on the entire cell. After superimposing the different optical sections the vertical projection of the cell can be obtained. The area and shape factors \( \phi \) (see section 2.2.2) of the fibroblasts cultured on all four surfaces were calculated from the images. Cell area on hydrophilic smooth surfaces was found statistically significantly different from the cell area on hydrophobic and hydrophilic SLA surfaces but not from the cell area on smooth hydrophobic surface (Bonferroni \( p<0.05 \), \( n=21 \) mean value ± standard deviation). However, the cell shape factor \( \phi \) was not statistically different among the four investigated surfaces. The results are presented in Fig. 3.2-8 and Fig. 3.2.9.

**Figure 3.2-8** Area measurements for fibroblast cultured on hydrophobic and hydrophilic smooth and SLA surfaces. Cells were seed at a concentration of \( 1.3 \times 10^4 \) cells/ml. and incubated for 24 h (\( n = 21 \) for each surface, mean value ± standard deviation).
As discussed above, the presence of serum tends to minimize the differences between the fibroblast behavior on hydrophobic and the hydrophilic surfaces within the same topography. Although SEM pictures suggest that more cells attach to the smooth surface, all area and spreading measurements show that cell spreading and area are similar on all four investigated surfaces. This result agrees with some reports found in literature [17] where the cell area and spreading was found similar in the presence of 15% fetal calf serum for surfaces with different surface energy. Others [18] report equivalent attachment of fibroblasts on chemically derivatized (SAM) surfaces, but found different stress fiber formation. Results on cell type-specific responses such as stress fiber formation will be presented in section 3.2.3.

Figure 3.2-9 Cell shape factors \( \phi \) of fibroblasts cultivated on hydrophobic and hydrophilic smooth and SLA surfaces (\( n = 21 \) for each surface, mean value \( \pm \) standard deviation)
3.2.1.2 Positively and negatively charged surfaces.

Pictures of fibroblasts seeded on positively and negatively charged SLA surfaces are presented in Fig. 3.2-10. Compared to reference SLA surface (Fig. 3.2-11), cells appear to cover more surface on negatively charged surfaces. Group of cells forming cluster (Fig. 3.2-12) were observed on all three investigated surfaces, with more clusters on the negatively charged surface. Similar cell morphology (of single cells) on all three surfaces was found at higher magnification.

Figure 3.2-10 Fibroblasts cultured on positively (a) and negatively (b) charged surface.
Figure 3.2-11 Fibroblasts cultured on reference SLA surface.

Figure 3.2-12 Cluster of fibroblasts cultured on negatively charged surface.
As for the hydrophilic and hydrophobic surfaces, confocal pictures were taken and used for cell area and cell shape factor determinations. Cell area are significant different between the positively and negatively charged surface (Bonferroni p<0.05, n=34 mean value ± standard deviation). Cell cultured on negatively charged surface shown a smaller cell area as cell cultured on positively charged surface. However, cell shape factor $\phi$ was found not to differ significantly for the three surfaces analyzed. Results are shown in Fig. 3.2-13 and 3.2-14.

**Figure 3.2-13** Cell area of fibroblasts cultured on reference, positively charged and negatively charged SLA surfaces (n = 34, mean value ± standard deviation).
One study found in literature [21] reports equivalent cell spreading and cell area for fibroblasts cultured on positively (amine- and quaternary amine-modifies glass) and negatively smooth charged surfaces (thiol- and oxidized thiol-modified glass). However, the differences in chemical composition and in topography between the surface used in that study and this one mitigate against meaningful comparisons.
3.2.2 Cell thickness

3.2.2.1 Hydrophilic and hydrophobic surfaces

The same samples used for the cell area and shape factor determination (section 3.2.1.1) were also used for cell thickness measurements using CLSM. Results of the cell thickness measurements after 24 h incubation at a cell concentration of $1.3 \times 10^4$ cells/ml are shown in Fig. 3.2-15.

![Figure 3.2-15](image-url)

**Figure 3.2-15** Fibroblasts thickness cultured on smooth and SLA surfaces (n = 8, mean value ± standard deviation).

Cells cultured on hydrophobic and hydrophilic SLA surfaces are significantly thicker than cells cultured on the corresponding smooth surfaces (Bonferroni p<0.05, n=20). This result is in accordance with previous studies [44]. Smooth surfaces offer more opportunities for a close contact between cell membrane and the smaller cell thickness found for fibroblasts on smooth surfaces could be explained by the presence of more focal adhesions on the cell membrane. It is well recognized that fibroblasts adhere more
efficiently on smooth surfaces [35], but no indication about a possible correlation between cell thickness and cell attachment could be found in literature. However, no statistically significant difference was found for cells thickness when the hydrophobic and hydrophilic surface were compared with the same topography.

3.2.2.2 Positively and negatively charged surfaces.

The thickness of fibroblasts seeded on positively charged, negatively charged and reference SLA surfaces is presented in Fig. 3.2-16. Cells cultured on negatively charged SLA surface were found to be statistically higher than cells cultured on positively charged SLA surface (Bonferroni p<0.05, n=19, mean value ± standard deviation).

![Figure 3.2-16](image)

**Figure 3.2-16** Fibroblasts thickness cultured on positively and negatively charged SLA surfaces (n = 19, mean value ± standard deviation). Reference surface represent SLA surfaces without SAM.
In contrast to the hydrophobic and hydrophilic SLA surfaces, electrical charge seems to have an influence on fibroblasts thickness on rough surfaces.

3.2.3 Cytoskeletal organization: actin staining

3.2.3.1 Hydrophilic and hydrophobic surfaces

Digital CLSM pictures were taken using a He-Ne laser (\(\lambda=540\) nm) to excite the fluorophore and a filter to select only the light emitted from the staining (\(\lambda=580\) nm) attached to the actin. For both smooth and SLA surfaces, cells were scanned at 3 to 8 different focus planes (all focus planes lying in the cell) with a difference between each plane of 1 \(\mu m\). Composite CLSM pictures are obtained by superimposing the focal plane at different heights. Fig 3.2-17 shows picture of the cells seed on smooth and SLA surfaces.

Figure 3.2-17 composite CLSM images of a fluorescent labeling of actin after a cultivation of 24 h on smooth hydrophilic (a), smooth hydrophobic (b), hydrophilic SLA
(c) and hydrophobic SLA surfaces (d). Images were obtained from three to eight sections (1 µm step size).

Actin stress fiber formations were more evident on smooth surfaces, while on SLA surfaces the cells present a more diffuse actin distribution. In some cells cultured on hydrophilic SLA surfaces, as well as in some cells cultured on hydrophobic SLA surface, cytoskeletal structures called actin edge-bundles (AEB, Fig. 3.2-17 c) were observed. AEB are specialized cytoskeletal structures composed by a single microfilament bundle following the outline of every webbed edge and support it [45]. The presence or the lack of these structures could be related to changes in cell metabolism and gene expression as a result of the change in cell shape and/or related to the contractile force being applied to the surface by the cell.

3.2.3.2 Positively and negatively charged surfaces.

The same procedure as for the actin visualization of fibroblasts seeded on hydrophobic and hydrophilic surfaces were also performed on positively and negatively charged surfaces. CLSM pictures presenting fibroblasts cytoskeletal organization are shown in Fig. 3.2-16.
Figure 3.2-18 composite CLSM images of a fluorescent labeling of actin after an cultivation of 24 h on reference SLA (a), positively charged SLA (b) and negatively charged SLA surface (c, d). Images were obtained from three to eight sections (1 µm step size).

Fibroblasts cultured on SLA surfaces do usually not show actin stress fibers formation. In most of the case, the actin was diffused in the cytoplasm as observed for the cells seeded on hydrophobic and hydrophilic SLA surfaces (section 3.2.3.1). Topography play also an important role in the actin stress fibers formation process. This is in accordance to recently results of in-vivo observations [46].

In some cases AEB were observed, especially on reference and on negatively charged SLA, but no attempt at quantification was made in this exploratory study. Fig. 3.2-18 d) shows an example of a fibroblast on negatively charged SLA with pronounced AEB. In contrast, one study found in literature reports no difference in the cytoskeletal organization for fibroblasts on surface with different electrical charge [21]. However, the surface chemical composition and the topography used in this work differ from the study mentioned above and therefore as already discussed in section 3.2.1.2, the cell behavior would not be expected to be identical.
4 Conclusion

The successfully surface modifications using the SAM technique were confirmed by XPS measurements. The functional groups of the adsorbed molecules forming the SAM were detected on both the smooth and rough (SLA) surfaces. However, further works have to be done to investigate the arrangements of the SAM, especially for the determination of the order (surface coverage) of the structure. As expected, chemically and topographically treated surfaces influence cells responses to the surface in different ways. First, the topography was found to play a major role in fibroblasts attachment and proliferation. In fact, rough surfaces were less covered by fibroblasts than smooth surfaces, when cultured for 24 hours in medium with 15% serum. Moreover, fibroblasts cultured on rough surfaces indicate smaller cell areas and are significant thicker compared to fibroblasts cultured on smooth surfaces. Rough surface topography also inhibits actin stress fiber formation: actin was detected by fluorescent staining in a diffuse form in the cytoplasm.

Second, surface chemistry and surface charge influence the cell behavior. In case of fibroblasts cultured with low serum content for 24h, cell spreading on the hydrophobic smooth surface was enhanced compared to cells spread on the hydrophobic smooth surface. However the difference become smaller with higher serum content. With a serum content of 15%, no statistical significant differences in terms of cell attachment, area, shape and thickness could be observed between hydrophilic and hydrophobic surfaces with the same topography. On the other hand, fibroblasts are influenced by the surface charge even in high serum condition (15%); cells cultured on the positively charged SLA surface show smaller area and bigger thickness compared to cell cultured on the negatively charged SLA surface.

Fibroblasts cultured on surfaces with very different advancing and receding CA (e.g. hydrophobic and hydrophilic SLA surfaces) did not show any differences, but show different behavior to surfaces with the same advancing and receding CA (e.g. positively charged and negatively charged SLA surfaces). Although, both CA measurements and fibroblast behavior are known to dependent on surface chemistry and topography, CA
surface characterizations alone do not necessary allow a prediction of fibroblasts responses to the surface. As suggested by the 0% serum condition, the role of the serum protein is very important and may prevent a direct relationship between CA measurements and fibroblast responses to the surface.

In order to estimate the possibility of a clinical application (e.g dental root implant) of the SAM modified surfaces used in this study, further investigations involving other cell types such as osteoblasts and epithelial cells are required. Moreover, it is well known that different cell populations react in different ways \textit{in-vitro} and \textit{in-vivo}. Predictions of the success or the failure of an implant can only be reliable if the reactions of all cell populations are known. However, competition between cell types and the different conditions between \textit{in-vitro} and \textit{in-vivo} tests may lead to biological responses to the implant surfaces that are different from the expected ones. Therefore, \textit{in-vivo} test are also needed for a complete evaluation of these SAM modified surfaces as a new titanium implant surface.
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6 References


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