Design and Application of Surface-Chemical Gradients for Biological Investigations

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For my family & friends

致
我亲爱的家人朋友
“The most beautiful experience we can have is the mysterious—the fundamental emotion which stands at the cradle of true art and true science.”

“Out of clutter find simplicity; From discord find harmony; In the middle of difficulty lies opportunity.”

“The most practical solution is a good theory.”
Abstract

Surface gradients constitute an advantageous platform, since they allow high-throughput and cost-effective experiments involving the exploration of a large variety of surface parameters for materials science, tribology and especially in biological studies (e.g. protein adsorption and cell response). This thesis is split into two separate projects related to the preparation and application of spatially controlled surface chemistry.

• For the first project, PEG-surface-density gradients were employed as screening tools for a systematic study of the influence of PEG chain density/conformation on protein adsorption, cell and bacterial adhesion behavior, as well as the correlation between them. These issues are central towards developing coatings for implants and related biomedical applications.

Briefly, gradients with a linear change in coverage of the polycationic polymer PLL(20)-g(3.5)-PEG(2) were prepared on titanium dioxide surfaces by a controlled dipping process, and characterized by variable-angle spectroscopic ellipsometry (VASE) and fluorescence microscopy (FM). To investigate the interactions of proteins, cells and bacteria as a function of PEG densities, a series of adsorption, adhesion and proliferation assays were carried out on PLL-g-PEG gradients.

For protein adsorption tests, the adsorption behavior of single proteins generally correlated with semi-empirical geometric models, illustrating the effect of the PEG-chain surface distribution on the inhibition of protein adsorption. Distinct differences could be observed between individual adsorbing proteins, attributable to their mode of surface attachment. The single and competitive adsorption of protein solutions containing albumin and fibrinogen was then investigated by fluorescence microscopy, indicating a larger amount of fibrinogen adsorption compared with albumin adsorption (in minutes to hours) along the entire PLL-g-PEG gradient samples.

For eukaryotic cell tests, to further elucidate the underlying mechanism of cell adhesion and spreading as a function of PEG coverage and the potential involvement of integrins, short-term cell-adhesion assays were carried out with human foreskin fibroblasts (hFF) and rat calvarial osteoblasts (RCO). Long-term proliferation and viability assays were also studied with hFF and RCO, and with different backfilling proteins (fibrinogen, albumin, fibronectin, serum) on PEG gradients.

For bacterial adhesion studies, similar assay conditions were applied. In physiological buffer, the adhesion behavior of both E.coli and S.aureus is similar to that of single-protein adsorption. Backfilling of proteins on PEG gradients decreased the initial bacterial
adhesion to different extents, depending on the type and amount of proteins, and also the strains of bacteria. The results are quite different from those of hFF and RCO.

The use of surface-gradient samples demonstrated the importance of PEG conformation, the amount of exposed titanium dioxide surface area (and its distribution), and the structure and chemistry of the proteins involved for protein adsorption. Correspondingly the influence of these factors on eukaryotic cell and bacterial adhesion could be directly observed. Insights were gained into the roles of both nonspecific binding and specific integrin binding in eukaryotic cell adhesion, and the differential adhesion between eukaryotic cell and bacteria possibly due to the physical (and bio)-chemical properties of the bacterial cell wall and eukaryotic cell membrane.

• The objective of the second project was to develop a reproducible preparation method for ultraflat, patterned surfaces of composite metals, and subsequently to combine current gradient techniques to ultimately produce more versatile surface (bio) chemical gradients platforms with a higher throughput than presently achieved.

Ti/Au patterned ultraflat surfaces were developed by a modified template stripping method. The generated sample surface can be as large as several square centimeters, was free from residues from the silicon-based template (silicon wafer in our case), was robust enough to tolerate common solvents such as water and ethanol and many experimental conditions, and worked well with different area ratios of Ti vs. Au. Briefly, silicon wafers were used as ultraflat templates. A standard photolithography process followed by metal deposition was applied to generate patterns of different metals. Additionally, wafers were subjected to silanization to reduce the adhesion between Ti and silicon surfaces. After gluing glass slides as supports, the ultraflat surfaces of Ti/Au pattern were achieved by mechanical stripping from silicon wafer templates. The generated sample surfaces were characterized by atomic force microscopy (AFM), time-of-flight secondary ion mass spectrometry (ToF-SIMS) and X-ray photoelectron spectroscopy (XPS).

Furthermore, the Ti and Au regions on the sample surfaces were independently chemically functionalized employing catechol-and thiol-based SAMs (self-assembled monolayers) systems, generating patterns of chemical differences without the interference of surface roughness. By incorporation of a gradient fabrication technique developed previously in our lab, the generated patterned ultraflat surfaces can be further modified as surface-chemical gradients. The characterization of chemical modification was performed by means of ToF-SIMS, XPS, microdroplet condensation and AFM.

Finally, preliminary applications of the generated samples were exhibited, including moving droplets on surface-wettability gradients in channels, and controlled localization of cell adhesion on the patterned surfaces.
Oberflächengradienten bieten gegenüber homogenen Oberflächen Vorteile, weil damit kostengünstige Hochdurchsatzexperimente durchgeführt werden können. In der Materialwissenschaft, der Tribologie und vor allem der Biologie (z.B. was die Proteinadsorption und die zelluläre Reaktion betrifft) können so eine grosse Anzahl Oberflächenparameter gleichzeitig untersucht werden. Diese Arbeit beinhaltet zwei Projekte, welche sich mit der Herstellung und Anwendung von Oberflächen mit räumlich kontrollierter Oberflächenchemie beschäftigen.

• Im ersten Projekt wurde mit Hilfe von PEG-Oberflächengradienten der Einfluss von Dichte und Konformation der PEG-Ketten auf die Proteinadsorption und die Adhäision von Zellen und Bakterien, sowie deren Korrelation untersucht. Diese Fragestellungen sind von zentraler Bedeutung, wenn Beschichtungen für Implantate oder ähnliche biomedizinische Anwendungen entwickelt werden sollen.

Mit Hilfe eines kontrollierten Eintauchprozesses wurden Gradienten hergestellt, welche eine linear zunehmende Bedeckung mit dem polykationischen Polymer PLL(20)-g(3.5)-PEG(2) auf Titanoxid-Oberflächen aufweisen. Die Charakterisierung erfolgte durch Ellipsometrie (variable-angle spectroscopic ellipsometry, VASE) und Fluoreszenzmikroskopie. Um die Interaktion von Proteinen, Zellen und Bakterien in Funktion der PEG Dichte zu untersuchen, wurde eine Reihe von Adsorptions-, Adhäsions- und Proliferationsexperimenten durchgeführt.

Für die Proteinadsorptionsexperimente korrelierte das Adsorptionsverhalten einzelner Proteine generell mit halb-empirischen geometrischen Modellen, was der Verteilung der PEG-Ketten auf der Oberfläche zuzuweisen ist. Es wurden deutliche Unterschiede zwischen einzelnen adsorbierten Proteinen beobachtet, welche auf deren Bindungsort zurückgeführt werden können. In der Folge wurden mit Fluoreszenzmikroskopie die individuelle und die kompetitive Adsorption von Albumin und Fibrinogen aus gemischten Lösungen untersucht. Diese Experimente zeigten, dass entlang des kompletten PLL-g-PEG Gradienten eine grössere Menge Fibrinogen als Albumin adsorbiert (in Minuten bis Stunden).

Kurzzeit-Zelladhäsions-Experimente mit Fibroblasten (human foreskin fibroblasts, hFF) und Osteoblasten (rat calvarial osteoblasts, RCO) wurden durchgeführt um aufzuklären, welcher Mechanismus der Adhäsion und der Ausbreitung der Zellen in Funktion der PEG Dichte zu Grunde liegt. Ausserdem wurde untersucht, in welcher Art Integrine möglicherweise beteiligt sind. Zusätzlich wurden auch Langzeit-Proliferations- und Viabilitätsexperimente mit hFF und RCO durchgeführt. Dazu wurden die PEG-
Gradienten mit verschiedenen Proteinen (Fibrinogen, Albumin, Fibronectin oder Serum) rückgefüllt und danach untersucht.


- Das Ziel des zweiten Projekts war es, eine reproduzierbare Methode zur Herstellung von ultraflachen, gemusterten Oberflächen von Verbundmetallen zu entwickeln, und anschliessend die gängigen Techniken zur Herstellung von Gradienten so zu kombinieren, dass eine noch vielseitigere (bio)chemische Plattform mit einem noch höheren Durchsatz entsteht.

Flugzeit-Sekundärionen-Massenspektrometrie (ToF-SIMS) und Röntgen-Photoelektronen-Spektroskopie (XPS) charakterisiert.

Des Weiteren wurden die Au und Ti Bereiche der Probenoberfläche unabhängig voneinander mit Hilfe von Thiol-, respektive Katechol-basierten SAMs-Systemen chemisch funktionalisiert. So konnte ein chemisches Muster hergestellt werden, ohne dass die Oberflächenrauhigkeit verändert wurde. Mit Hilfe einer früher in unserem Labor entwickelten Methode zur Herstellung von Gradienten konnten die gemusterten, ultraflachen Oberflächen weiter zu chemischen Oberflächengradienten entwickelt werden. Die Charakterisierung der chemischen Modifikation erfolgt mit ToF-SIMS, XPS, der Kondensation von Mikrotropfen ("microdroplet condensation") und AFM.

Zum Schluss wurden erste Anwendungen der hergestellten Proben gezeigt, wozu in Kanälen und auf Benetbarkeitsgradienten "wandernde" Tropfen, oder die kontrollierte Eingrenzung der Zelladhäsion auf gemusterten Oberflächen gehören.
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Introduction

This chapter introduces the concept of spatial control of surfaces, including surface-chemical gradients and patterned surfaces, and provides an overview of the current techniques and applications. The scope of the thesis and an outline of the thesis chapters are given at the end.

1.1 General Introduction

Homogeneity is not always good. Neither in our daily lives, nor in science! In fact, nature is full of heterogeneity. Although the majority of surface scientists strive to work with “homogeneous” samples, many specific applications profit from heterogeneity and the advantages that it can offer.

In the field of surface science and technology, fabricating and utilizing heterogeneous samples to achieve spatial control of surfaces has been an active research area in recent decades. Different functionalizations are laterally distributed on the surfaces, and depending on the subsequent applications, parameters such as length scale, directionality, dimensionality and continuity of the heterogeneity come into play. For instance, the length scale should be correlated to the size of the interacting objects, such as water droplets, colloids, or cells.

1.1.1 Patterned Surfaces

If arrays of contrasting structures consisting of more than one functionality are present, the surface is considered to be patterned. Surface patterning is of foremost importance in various areas of modern science and technology, with implications ranging from information-storage devices, micro- and nano-electromechanical systems (MEMS and NEMS) to bioengineering, biological studies and studies of various surface and interfacial physiochemical phenomena, such as wetting.
The feature size of the functionality is probably the most important issue to be considered. Depending on the length scale of the probing systems and requirements for subsequent applications, nowadays there are many different of techniques that one can choose from, as illustrated in Fig. 1.1. The principle of all these modern techniques actually can be referred to as lithography, which was first invented in 1796 as an economical means to publish theatrical works [1]. It typically begins with pre-designed pattern structures in a master or mask and ends up with arrays of patterns on substrates, employing certain means to transfer or replicate the structures. Among these techniques, micro- and nano-scale methods have been playing the most important role in recent decades, and still remain active research areas.

A commonly used and simple micrometer/sub-micrometer-scale patterning technique is known as “microcontact printing (µCP)”. It is a form of soft lithography, first introduced by the Whitesides group in Harvard in 1993 [2,3], which use a topographically structured master PDMS stamp to transfer molecular inks onto substrates through conformal contact. However, it has some limitations including practical problems such as pattern homogeneity, stamp deformation, surface contamination, ink mobility etc [4,5].

![Diagram of patterning techniques on different length scales](image)

**Fig. 1.1** Patterning techniques on different length scales, compared to the sizes of various objects [6].
Another extensively used method is photolithography, which is a common process in microfabrication, involving the selective removal of part of a thin film, and sharing some fundamental principals with photography. Briefly, the process relies on light to transfer pattern structures from optical masks to light (UV light in most cases)-sensitive chemicals, known as “photo-resists”, that have been deposited on substrates. Subsequently, either deposition of a new material (i.e. metal film) or chemical modification on the desired patterns is carried out. A typical photolithography process is shown is Fig 1.2(a). More details about the basic principle, set-up and experimental conditions can be found in [7]. The previously well-developed patterning techniques for cell-surface interaction studies in our group “MAPL (Molecular Assembly Patterning by Lift-off)” by Falconnet et al. [8] and “SMAP (Selective Molecular Assembly Patterning)” by Michel et al. are both based on photolithography [9].

![Photolithography Process](image)

**Fig. 1.2** (a) An illustration of a typical photolithography process. (b) Molecular-assembly patterning by lift-off (MAPL). The photoresist-patterned sample ultimately reveals a molecular contrast of PLL-g-PEG, non-adhesive patches versus interactive patches [8]. (c) A schematic illustration of the SMAP methodology, which generates a material contrast of TiO$_2$ squares within the SiO$_2$ matrix, and further selective functionalization with DDP on TiO$_2$, protein on DDP, and PLL-g-PEG on SiO$_2$ [9].

The nano-scale patterning techniques follow the same concepts as micro-scale fabrication. The past few years have witnessed great advances in new methods and technologies, such as conventional nanoimprint lithography [10], e-beam lithography [11], optical-based interference lithography [12], and probe-tip-based lithography (i.e. nano-shaving, dip-pen) [13,14]. Beside these conventional lithography methods,
particle lithography is another quite attractive approach, as it is simple and inexpensive, and can achieve ordered nanostructures over relatively large areas (up to \( \text{mm}^2 \) or even \( \text{cm}^2 \)). One simple, scalable, single-step approach, recently developed in our group, employs colloidal self-assembly at liquid-liquid interfaces (SALI) to fabricate non-close-packed particle masks. It has been shown that this approach successfully applies to a wide range of particle size (40-500 nm) and their separation can be controlled independently between 3 and 10 particle diameters [15].

### 1.1.2 Surface-chemical Gradients

One of important applications and advantages of surface patterning lies in the high-throughput and cost-effective measurements it facilitates. This is especially the case for biological applications, where the complexity of cell-surface interactions present difficulties in isolating and ultimately identifying the importance of specific biomolecular or chemical properties. The widespread and rapid proliferation of various micro/nano-patterning techniques indicates, to some extent, the urgent need to develop these tools for analysis and screening purposes. Surface gradients—structures generally defined as molecular patterns with a gradual change of one or more characteristics, also serve as an important subset of high-throughput methodologies.

In recent years, gradients have played an important role in investigating a whole host of surface-related phenomena [16-18]. By using a single gradient sample instead of many individual samples, the entire range of certain particular parameters can be studied under the same experimental conditions, resulting in the minimal amount of systematic errors, repetitions, time and material. Besides these significant advantages, another motivation for fabricating gradients relies on the ubiquity of gradient phenomena in the life sciences, e.g. chemotaxis of cells and bacteria. For these reasons, biomimetic gradients provide an efficient and valuable platform to gain a systematic and deep insight into biological processes \textit{in vitro}.

Through the use of a variety of surface-chemical strategies, changes in certain surface properties ((bio)chemical or physical) can be controlled to gradually change over a given distance, resulting in a surface gradient. The preparation of surface-chemical gradients has mainly involved two approaches: self-assembled monolayers (SAMs, e.g. alkanethiols) and polymer coatings (e.g. PEG). A large variety of methods have been employed to fabricate surface-chemical gradients, which can be simply divided into two categories: “top down” and “bottom up”.
One commonly used technique for generating chemical gradients was first developed in 1992 by Chaudhury et al. involves the diffusion of silanes across a solid substrate [19]. Wettability gradients prepared in this way, were used to move water droplets uphill, using the surface-energy slope to counteract gravity. Brush-like polymer gradients can also be prepared by this technique through additional modification, either by applying a “grafting from” method to the gradient of silane initiators with monomers [20], or through the direct attachment of polymer chains to active sites of silane gradients—a “grafting to” method.

Subsequently, a large number of other methods have been developed to create a variety of surface-chemical gradients, varying in dimensionality, directionality, length scale, time dependency and functionality. Genzer et al. [22] and Morgenthaler et al. [23] have separately summarized the different approaches for gradient preparation and their applications as listed below in Fig. 1.4 and Table 1.1.

In our group, a simple and reproducible approach was previously developed by Morgenthaler et al. and successfully used in producing chemical gradients over a wide compositional range and over a distance of centimeters [24]. It applies to fabricating not only SAMs gradients but also polymer-brush gradients.

The approach was initially used to produce a surface concentration gradient of methyl- and hydroxyl-terminated thiolates on gold substrates, and it involves a slow
dipping process controlled by a linear-motion drive. Surface wettability can be changed linearly along this alkanethiol gradient composed of –CH$_3$ and –OH terminated thiols.

Later this technically simple approach was employed to generate a PLL-g-PEG surface-density gradient [25]. It is based on adsorbing a positively charged PLL backbone onto the negatively charged metal oxide substrate (TiO$_2$ or Nb$_2$O$_5$) in a physiological buffer solution by means of electrostatic interactions.

<table>
<thead>
<tr>
<th>Technique</th>
<th>Adsorbate</th>
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<th>Application</th>
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<tr>
<td>Diffusion</td>
<td>Vapor phase</td>
<td>Silanes</td>
<td>Si, PDMS</td>
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<td>Solvent</td>
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<td>Alkanethiols, polymer</td>
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<td>Printing</td>
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<td>Biomolecules</td>
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<td>Proteins</td>
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<td>Desorption</td>
<td>By potential</td>
<td>Alkanethiols, polymers</td>
<td>Au</td>
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<td>Advancing solution</td>
<td>Concentration</td>
<td>Proteins</td>
<td>Glass, Si, Au</td>
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<td>gradient</td>
<td>Monomers</td>
<td>Funct. Si</td>
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<td>Controlling reaction time</td>
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<td>Proteins, biomolecules</td>
<td>Funct. Si, filter</td>
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<td>Silane</td>
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<td>Irradiation</td>
<td>Depletion</td>
<td>Alkanethiols</td>
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<td>Proteins</td>
<td>PVC, PDMS, glass</td>
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<td>Change exposure</td>
<td>Polyanionic ions</td>
<td>PMMA, Polymer</td>
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<td>Temperature</td>
<td>time</td>
<td>Monomer solution</td>
<td>Polymer, Funct. Si</td>
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<td>Through mask</td>
<td>Proteins</td>
<td>Funct. PS, Si, nanoporous Si</td>
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<td></td>
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<td>Silane</td>
<td>Si, nanoporous Si</td>
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<td>Through mask</td>
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<td>Plasma polymerisation</td>
<td>Alkanethiols</td>
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<td>Electropolymerisation</td>
<td>(pot. gradient)</td>
<td>Polymer</td>
<td>Au</td>
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Table 1.1 Surface-chemical gradient preparation techniques, systems and their applications [22].
Fig. 1.4 An overview of various methods and techniques of creating molecular and macromolecular gradients [21].
1.2 Scope of the Thesis

The aim of this thesis is two-fold. On the one hand, well-developed PLL-g-PEG-density gradients are used for fundamental protein-adsorption, cell- and bacterial-adhesion studies, helping to gain insight into the adsorption and adhesion mechanisms involved when biological systems encounter materials surfaces, and thus allowing for tuning adhesion in implant-related applications.

On the other hand, we aim at spanning the realm of spatially controlled surfaces, in particularly, to generate model surfaces of chemical heterogeneity but without influence of roughness and topography, for fundamental research and other applications. This part of work focused on the combination of a modified template-stripping method with established photolithography, in order to achieve Ti/Au-patterned ultraflat surfaces. Furthermore, by applying thiol-based and catechol-based SAMs chemistries, spatial control of different properties (i.e. wettability and anti-fouling) can be achieved.

1.3 Outline of Thesis Chapters

The rest of this thesis is divided into two parts with respect to two major projects.

Chapter 2 Surface-characterization and bio-analytical methods employed in the two major projects

From Chapter 3 to Chapter 7, biological studies involving the use of surface-chemical gradients are described:

Chapter 3 Principles and experimental conditions necessary to fabricate PLL-g-PEG gradients by a simple dipping process, followed by experimental characterization and theoretical considerations.

Chapter 4 Protein-adsorption studies involving the use of generated PLL-g-PEG gradients, including single-protein adsorption and competitive adsorption.

Chapter 5 Eukaryotic cell (hFF and RCO) adhesion studies involving the use of PLL-g-PEG gradients, including short-term adhesion, long-term adhesion and proliferation, and viability assays.
Chapter 6 Bacterial adhesion studies using PLL-g-PEG gradients. Results are discussed in comparison with eukaryotic cell adhesion.

Chapter 7 Conclusion and outlook of the first project

From Chapter 8 to Chapter 10, the second project regarding patterned ultraflat surfaces is described.

Chapter 8 Fabrication technique for patterned ultraflat surfaces

Chapter 9 Chemical functionalization of patterned ultraflat surfaces, in particular, with catechol-based and thiol-based chemistries, and combination of the chemical-gradient technique with catechol-based and thiol-based chemistries

Chapter 10 Conclusion and outlook of the second project
A brief overview of all the surface chemical and topographical characterization techniques employed in the two major projects is provided, from basic working principles, through instrumentation, to applications, data acquisition and analysis. Finally, a summary of the most widely used surface bioanalytical method—immunohistochemical staining (IHC)—is added to assist the reader who is not a biologist.

### 2.1 Optical Waveguide Lightmode Spectroscopy (OWLS)

Optical Waveguide Lightmode Spectroscopy (OWLS) [26] is a label-free sensor technique allowing *in-situ* monitoring of adsorption, desorption, adhesion and biospecific binding events. The principle of this technique is based on linearly polarized laser light coupled into a planar waveguide via an optical grating at two specific well-defined incident angles. The light in the waveguide propagates distinctly as transverse electric (TE) and transverse magnetic (TM) modes, and for each polarization mode, there is an incoupling angle corresponding to the maximum constructive interference.

The incoupling angles are sensitive to changes in the refractive index within the evanescent field above the surface of the waveguide. Therefore by continuous measurement of the shift of the incoupling angles it enables determination of the adsorbed mass and the number of adsorbed molecules using de Feijter’s formula

$$M = d_A \frac{n_A - n_C}{dn/dc}$$

Eq. (2.1)

where $d_A$ is the “dry” thickness of the adlayer and $dn/dc$ is the refractive index increment of the molecules, which may be measured using a refractometer. The adsorbed mass density determined from Eq. (2.1) depends only on the difference in the refractive index of the adsorbates ($n_A$) and the covering medium ($n_C$), thus the
coupled solvent molecules will not contribute to the mass.

This technique has a high sensitivity of several ng/cm$^2$ up to a few hundred of nanometers above waveguide surfaces. Besides, a measurement time resolution of a few seconds allows for an *in-situ*, real-time monitoring of adsorption kinetics.

![Fig. 2.1 Schematic drawing of set-up of an OWLS experiment. [27]](image)

### 2.2 Variable-Angle Spectroscopic Ellipsometry (VASE)

Ellipsometry is an optical technique relying on the external reflection of a light beam from a reflective surface, where circularly-polarized light is applied. From the changes of polarization of light, the optical properties, thickness of adlayers or films, morphology on the surface can be calculated, and can be used to determine i.e. the amount of adsorbed molecules on a surface. It is extensively used from basic research to industrial applications to measure accurate thicknesses of films ranging from a few angstroms to several micrometers with excellent accuracy of a few angstroms. Furthermore, as an optical technique, it is contactless and non-destructive.

VASE [28] measures at different angles of incidence. The typical range for spectroscopic ellipsometry measurements is 50° to 75°. While standard measurements of thickness and refractive index require only a single angle of incidence, multiple angles of incidence are necessary for very thick films (> 1 µm) and complex multilayers, and are advantageous for samples that do not require multiple angles of incidence, as extra angles can confirm that the chosen optical model is unique.

A standard spectroscopic ellipsometer consists of a light source to generate light beam of various of wave lengths, a polarizer to provide different states of polarization
by varying the angle of polarizer, a compensator (optional), and a second polarizer called analyzer for the light beam after reflected off the layer of sample, and a detector. The angles of the polarizer and analyzer are changed until a minimal signal is detected, meaning only light with the polarization perpendicular to the incoming polarization is allowed to pass. Therefore the angle of the analyzer is correlated to the direction of polarization of the reflected light. The measured two values of ellipsometry are expressed as $\Psi$ and $\Delta$.

\[
\tan(\Psi)e^{i\Delta} = \frac{R_p}{R_s} \tag{2.2}
\]

where $\Psi$ is the amplitude ratio of reflection and $\Delta$ is the phase shift. $R_p$ and $R_s$ represent for the amplitudes of $p$- and $s$-polarized light after reflection and normalized to their initial value, respectively. As ellipsometry measures the ratio of these two values, results can be very accurate and reproducible.

![Figure 2.2 Schematic setup of a VASE experiment.](image)

Typically, VASE data is acquired versus wavelength and angle of incidence of light beam. To generate the results information, an optical model needs to be built up, describing all layers structure using as much information as possible. Subsequently by comparing generated theoretical data from the optical model to experimental data, unknown parameters in the optical model (e.g. optical constants and/or film thickness) are varied to try and obtain a “best fit” to the experimental data.

Thickness measurements are not independent of optical constants for very thin
films (<50nm). For transparent material layers, often, a simple and empirical equation can be used to describe refractive index—the Cauchy model:

\[ n(\lambda) = A + \frac{B}{\lambda^2} + \frac{C}{\lambda^4} \]  

Eq. (2.3)

Where \( n \) is the layer refractive index, \( \lambda \) is the wavelength of the incident light, and A, B, C are coefficients can be determined by fitting the equation.

### 2.3 Atomic Force Microscopy (AFM)

AFM [29] is a very high-resolution type of scanning probe microscopy used to image or measure properties of chemical, material, biological surfaces. It consists of a cantilever (typically silicon or silicon nitride) attached with a sharp tip (probe) at its end to scan a sample surface. When the tip is approaching a surface, forces between tip and sample surface result in a deflection of the cantilever, which is measured using a laser spot reflected from the top face of the cantilever into a quad photodetector. The difference between the signals of the top and bottom photodiodes provides vertical deflection, revealing topographic features of a sample surface, while the difference between left and right is related to the degree of cantilever twisting and hence to the friction force.

![AFM signal: (A+B)-(C+D) FFM signal: (A+C)-(B+D)](image)

Fig. 2.3 Schematic of AFM working principle.
There are two common operating modes for imaging: contact mode and tapping mode.

In contact mode, as the name implies, the cantilever with a sharp tip of low spring constant, is “dragged” across sample surface. A low force (∼ nN) is maintained on the cantilever, thus pushing the tip against the sample surface as it is rastered under the action of a piezoelectric actuator. In constant-force mode, a feedback loop maintains constant set-point deflection by moving the scanner in the z direction. Thus, the vertical distance the scanner moves can be measured as a function of x and y coordinates and then converted into the topographic image of sample surface. In constant-height mode, the height of tip above sample surface is fixed and the deflection of cantilever is recorded.

In tapping mode, the cantilever instead is oscillated at a frequency near its resonant frequency, and the oscillating tip is then moved toward the surface until it begins to lightly tap the surface. Then the force between the tip and sample surface tends to influence the tip oscillation with respect to amplitude, frequency, and phase. This change in tip oscillation combined with the feedback loop system maintains a constant oscillation amplitude or frequency by adjusting the average tip-to-sample distance. Thus measuring the tip-to-sample distance as a function of x and y coordinates allows construction of the topographic image of a sample surface. Compared to contact mode, it does not lead to as much tip or sample surface degradation, which is especially beneficial for biological systems.

Compared to most optical or electron microscopy techniques, AFM provides a number of advantages. It enables the presentation of three-dimensional images of a sample surface at a very high resolution (nanometer to Ångström). Besides, it requires neither a vacuum environment nor any special sample preparation, and can be used in either ambient or liquid environments. Beyond topographic imaging, AFM is capable of phase imaging, friction and adhesion force measurements, nano-indentation and a variety of extended applications such as dip-pen lithography.
2.4 Time-of-Flight Secondary Ion Mass Spectrometry (ToF-SIMS)

ToF-SIMS [30] is a kind of mass spectrometry that is capable of identifying an analyte sputtered from the sample surface by measuring its molecular weight. Numerous different kinds of particle are emitted when primary ions or ion clusters bombard a sample surface, including a small portion of charged clusters. These generated charged clusters, termed “secondary ions”, are extracted from the surface by electrostatic means and allowed to fly through the flight path of an analyzer in ultra-high vacuum. By measuring the time they take to fly the known distance, according to the equation below, can then identify these fragments:

$$ t = L \times \sqrt{\frac{m}{2qU}} $$

where $t$ is the flight time, $L$ is the length of flight path, $m$ is the mass of fragments and $U$ is the applied voltage.

![Schematic diagram of ToF-SIMS instrument set-up and working principle.](image)

**Fig. 2.4** Schematic diagram of ToF-SIMS instrument set-up and working principle.

The measurements of flight time is carried by the timing electronics by applying a pulse of voltage to accelerate the primary ions and measuring the time between these pulses, and the electrical pulses when the ions reach the detector located at the end of the flight path. Fig. 2.4 shows the set-up of a typical ToF-SIMS instrument.

ToF-SIMS provides detection of all elements and isotopes, and chemical information via molecular and cluster ions. This generated information is highly
surface-specific, typically arising from the uppermost 1-2 nm of sample surface. Furthermore, it offers extreme sensitivity in the ppm to ppb range for most elements and sub-fmol/cm$^2$ for molecules, high mass resolution (>10000 m/$\Delta m$), high lateral resolution (sub-$\mu$m) as well as high-speed data acquisition. This technique is considered “non-destructive” in static mode provided that the primary-ion dose applied is maintained sufficiently low (<10$^{13}$/cm$^2$) to ensure that surface damage remains below 1% of a monolayer. Besides this so-called static SIMS mode, widely used for the characterization of molecular surfaces, ToF-SIMS instruments offer depth-profiling capability, so-called dynamic SIMS mode, which allows analysis of chemical layering by sequential sputtering of sample surfaces with a depth resolution better than 1nm.

There are three major operation modes of primary ion sources, depending on different applications. The differences in the spectra obtained are displayed in Fig. 2.5.

Usually, for mass spectra, to achieve increasing pulsed ion beam current while obtaining the highest possible mass resolution, a bunching of a short-pulsed ion beam (<1 ns) is often applied. This is so-called “high-current bunched” operation mode. It ensures high-mass resolution data acquisition without concurrent loss of counts, but at the expense of relatively low lateral resolution (ca. 5 $\mu$m).

In contrast, the so-called “burst-alignment” operation mode is suitable for obtaining the highest lateral resolution (sub-$\mu$m) for chemical imaging of surfaces (e.g. micropatterned surfaces) by using non-bunched primary ions. Basically, it works by focusing the primary ion beam to a narrow diameter via apertures and scanning it across the surface. A complete mass spectrum is obtained at each pixel in the raster of the ion beam. After data acquisition, a specific ion or a combination of ions can be selected by their mass-to-charge peaks from the spectrum and then presented as surface-distribution maps. But as the primary ion pulses cannot be bunched, and the pulse duration is much longer, it yields only a moderate mass resolution.

To obtain both good mass and spatial resolution (although not as good as for “bunched” and “burst alignment”, respectively), a compromise mode can be used, in which a short, unbunched pulse (~1.5 ns) is used, thus maintaining the spatial resolution. However, the price here is the count rate, and it is not generally available on all instruments. This is the so-called “burst” mode.
Fig. 2.5 Differences of spectra obtained in different operation modes of primary ion sources for different purposes. (a) in “high-current bunched” mode; (b) in “burst-alignment” mode; and (c) in “burst” mode. [30]

TOF-SIMS generates a lot of information, which sometimes can be overwhelming and extremely complicated, and therefore the interpretation of results can be time-consuming and cost-ineffective. There are a few simple approaches in some cases, i.e. recognition of fingerprint fragmentation of the molecules. Additionally, principal component analysis (PCA) has been the most commonly applied analysis technique, and is a multivariate analysis method that looks at the variance patterns within a data set to find the directions of greatest variance.

There are several limitations to ToF-SIMS measurements. Firstly, as with XPS, ToF-SIMS measurement is an ultrahigh-vacuum analysis technique, which necessitates dry and non-degassing samples. Secondly, unlike XPS, quantitative analysis is very difficult in ToF-SIMS due to matrix effects, instrumental drift, surface sensitivity and other factors. Lastly, ToF-SIMS is more sensitive to topography than XPS due to the working principle, and thus lateral chemical imaging of a high-topography surface is tricky and results can be misleading. Correlation with AFM is a potential way to solve this problem.

2.5 X-ray Photoelectron Spectroscopy (XPS)

Among all the contemporary surface characterization methods, X-ray photoelectron spectroscopy (XPS) [31], also known as ESCA, is most extensively used. The popularity relies on its sound theoretical basis, its high information content and the flexibility in addressing a wide range of sample species. As the name implies, it is based on the photoelectron effect on sample surface upon the irradiation of a beam of X-rays. Provided that the energy of incoming photons is high enough to overcome the work function of the material, core-level electrons then can be emitted from the
surface. The emitted electrons are sent through an analyzer (usually hemispherical), which utilizes an electric field to force electrons to follow different flight paths according to their velocities. The number of electrons with a given kinetic energy is then measured for the detected electrons, which are all emitted from the top ca. 10 nm of sample surface. The basic physics of the process can be described by the Einstein equation, simply expressed as:

\[ E_{B} = h\nu - E_{kin} - \Phi \]  

Eq. (2.5)

Where \( h\nu \) is the energy of X-ray source, \( E_{B} \) is the binding energy of the emitted electrons in the atoms, \( E_{kin} \) is the kinetic energy of the emitted electrons, and \( \Phi \) the spectrometer work function. Thus, the binding energy \( E_{B} \), indication of different elements and also their chemical or electronic state of each element, can be easily obtained from known values (\( h\nu \) and \( \Phi \)) and a measured value (\( E_{kin} \)). A typical XPS spectrum is presented in the form of number of emitted electrons detected (y axis) vs. binding energy (x axis).

![Schematic of the basic physics of XPS.](image)

**Fig. 2.6** Schematic of the basic physics of XPS.

In general, the binding energies of the photoelectrons are characteristic of the element from which they are emitted so that the spectra can be used for surface elemental analysis. Small shifts in the elemental binding energies provide information about the chemical state of the elements on the surface. Therefore, high-resolution XPS studies can provide chemical-state information for the surface. XPS thus provides not only results to differentiate different elements by a survey scan in minutes, but also can reveal chemical-state differences by high energy-resolution scans. Furthermore, quantitative analysis is possible, provided instrument- and element-related corrections, with a high accuracy of ca. 10% under optimum conditions (Eq. 2.6).
\[ N_A = \frac{I_A}{\sigma_A T_A \lambda_A \sin \theta} \]  

Eq. (2.6)

where \( I_A \) is the peak area of element A, \( \sigma_A \) is the photoionization cross-section of element A, also called sensitivity factors, \( \lambda_A \) is the inelastic mean free path, \( T_A \) is the transmission function of analyzer, and \( \theta \) is the take-off angle of the photoelectrons detected with respect to the surface normal.

In addition, XPS is also capable of lateral imaging of elemental composition either by using a focused X-ray beam to perform a raster scan over the sample surfaces, the rastering of lenses on the inlet to the analyzer, or through the use of a scanning sample stage. Depth profiling with XPS is also available by applying ion-beam etching or angle-resolved measurements, the latter being non-destructive.

![Schematic drawing of XPS set-up and working principle.](image-url)

**Fig. 2.7** Schematic drawing of XPS set-up and working principle.

### 2.6 Microdroplet-Density Measurement (µdd)

When cooling a sample in a vapor-enriched atmosphere of a substance that is liquid at room temperature (e.g. water), it tends to condense on the sample surface and nucleate in microdroplets—the same phenomenon that allows us to see our breath on cold days. The growing droplets can be assessed by counting the droplet density (droplet per \( \text{mm}^2 \)) in the “condensation figures” on sample surfaces at a specific time.
after nucleation starts. This is the so-called microdroplet density measurement (μdd) [32].

A simple set-up involves a transparent analysis chamber equipped with a sample mounting stage that can be cooled down by an ice-water mixture, for example. The condensation figures may be imaged using a CCD-camera coupled to a light microscope and analyzed by image-processing software.

This simple yet straightforward method provides qualitative and semi-quantitative information on homogeneity/heterogeneity of surface properties related to wettability, roughness, polarity, defects or order on a macroscopic to microscopic scale since water droplets nucleate more easily on hydrophilic, rough, and polar surfaces.

2.7 Contact-Angle Measurements (CA)

Contact angle is normally defined as the angle at which a liquid/vapor interface meets a solid phase. Thus it is determined by the thermodynamic equilibrium between the three interfaces, which can be described by Young’s Equation:

\[
\gamma_{SV} = \gamma_{SL} + \gamma_{LV} \cos \theta
\]

Eq. (2.7)

where \(\gamma_{SL}\), \(\gamma_{LV}\), \(\gamma_{SV}\) are the interfacial tensions between the solid and the liquid, the liquid and the vapor, and the solid and the vapor, respectively. The equilibrium contact angle that the liquid drop makes with the solid surface is denoted by \(\theta\).

![Schematic of basic physics of contact angle.](image)

**Fig. 2.8** Schematic of basic physics of contact angle.

Contact angle is often measured by the sessile-drop method, with a contact angle goniometer equipped with an optical system, by producing an appropriate drop volume on the tip of a syringe needle, which is then brought into contact with the solid surface. The static (equilibrium) contact angle is then measured after the drop detaches from syringe and spreads on the surface.
Contact angle can also be measured dynamically, which commonly involves a drop expansion/contraction method. It determines the largest contact angle possible without increasing its solid/liquid interfacial area by adding volume dynamically. This maximum angle is the advancing angle. Volume is removed to produce the smallest possible angle, the receding angle. The difference between the advancing and receding angle is the contact-angle hysteresis. Although the mechanism of hysteresis has not been fully understood, it is usually attributed to chemical heterogeneities and surface roughness.

Measuring contact angle is an easy and accurate way to determine surface free energy, if a series of different solvents are applied. It offers an easy-to-measure indication of the chemical and/or topographical properties of a solid surface, which are often related to wettability and adhesion, and also allows prediction of coating properties and detection of trace surface contaminants.

### 2.8 Fluorescence Microscopy (FM)

A fluorescence microscope [33] is an optical instrument used to image materials using the phenomena of fluorescence and phosphorescence. The specimen is illuminated with light of a specific wavelength through the use of an excitation filter. The energy is absorbed by the fluorophores, causing them to emit light of longer wavelengths. The illumination light is then separated from the much weaker emitted fluorescence through the use of a barrier filter. A typical fluorescence microscope consists of a light source (xenon lamp or mercury-vapor lamp), excitation filter that can be adjusted to the excitation wavelength range of fluorophores labeled with the specimen, dichroic mirror (or beamsplitter), and barrier filter to match the emission wavelength range of fluorophores (see Fig. 2.9 below).

Epi-fluorescence microscopy is the most extensively used FM technique, especially in the life sciences. The excitation light is passed through the objective lens and then onto the specimen, instead of directly through the specimen first. One major disadvantage of conventional widefield epi-fluorescence microscopy lies in the high background signal from outside the focal plane. Confocal fluorescence microscopy and TIRFM (Total Internal Reflection Fluorescence Microscopy) are two advanced optical imaging techniques that can be used to reduce the background signal to a large extent, as the depth of focal plane can be quite narrow (ca. 100 nm) above the specimen surface or at the specimen/medium interface.
In life sciences, fluorescence microscopy is a powerful tool allowing for accurate and sensitive detection of the distribution of proteins, DNA and other molecules of interest. Therefore, various of techniques have been developed for fluorescent staining and labeling of biological samples, which are summarized in the following section.

### 2.9 Immunohistochemical Staining and Immunofluorescence Labeling

There are a variety of methodologies that have been developed for analysis of cells and their activities, such as flow cytometry, immunoblotting, colorimetric-based assays and so on. Among these, immunohistochemical staining (IHC) [34] allows for *in-situ* characterization, and the results could be qualitative and quantitative. Immunofluorescence labeling (IF) [35] is a widely used example of IHC that makes use of fluorophores to visualise the distribution of the target molecules. Some of the fluorescent stains are small molecules which are intrinsically fluorescent upon binding a biological molecule of interest. In most common cases, an antibody is conjugated to a fluorophore, such as FITC or TRITC. Another advantage of IF is that, by choosing different conjugated fluorophores, multiple staining and subsequent multicolor visualization of different relevant targets in one slide could be possible. This increases the information obtained from each slide and reduces turnaround-time compared to single staining, and makes it possible to assess the topographic
relationship of the targets. So far many fluorescent stains have been designed for a wide range of biological molecules, some of which applied in our assays are summarized as below.

DAPI- 4',6-diamidino-2-phenylindole is a fluorescent stain that binds strongly to A-T rich regions in DNA. It is often used to stain fixed cells, as it passes through the membrane less efficiently in live cells. Once bound to DNA, DAPI has an absorption maximum at a wavelength of 358 nm and its emission maximum is at 461 nm (blue). Therefore for fluorescence microscopy, DAPI is excited with UV light and is detected through a blue filter. DAPI is extensively used for indication of nuclei of eukaryotic cells.

Hoechst- Hoechst dyes are bis-benzimidines. Similar to DAPI in that it is also a DNA stain with a preference for A-T rich sequences, which is compatible with both live- and fixed-cell applications. The dyes Hoechst 33258 and Hoechst 33342 are the ones most commonly used and they have similar excitation/emission spectra, excited by UV light at a wavelength of around 350 nm, and emitting blue fluorescent light (461 nm). Hoechst dyes are cell-permeable so they can be used for staining live cells. Besides, they are less toxic than DAPI, which ensures a higher viability of stained cells.

Phalloidin- A bicyclic heptapeptide, which binds F-actin filaments in high affinity. This property makes it a more active tool than actin antibodies for investigating the distribution of F-actin in eukaryotic cells by labeling phalloidin with fluorophores. Phalloidin does not permeate cell membranes and is toxic, making it less effective in experiments with live cells.

Live/Dead staining- FDA/EtBr staining. Once fluorescein diacetate (FDA) diffuses into cells, it becomes fluorescein after hydrolysis by nonspecific esterases in the cell cytoplasm. This causes live cells to fluoresce green under blue-light excitation (490 nm). Therefore, FDA fluorescence requires cellular esterase activity, which is an indication of cell viability. Permeability of the cytoplasmic membrane is commonly exploited to mark cells that are moribund or dead. Ethidium bromide (EtBr) stain penetrates only damaged, permeable membranes of nonviable cells that lose membrane integrity, and then binds covalently to their nucleic acids. This makes dead cells fluoresce orange.

ALP staining- Alkaline phosphatases (ALP) are a group of enzymes found primarily in liver and bone. ALP activity is an early marker of differentiation of cells
of the osteogenic lineage. A monoclonal primary antibody against ALP followed by fluorescently labeled secondary antibody was tested in our studies.

Osteocalcin staining- Osteocalcin is a noncollagenous protein found in bone and dentin. Osteocalcin secretion is a late marker of osteoblastic differentiation. A monoclonal primary antibody against osteocalcin followed by fluorescently labeled secondary antibody was tested in our studies.

Osteopontin staining- Osteopontin, also known as bone sialoprotein. Intracellular osteopontin expression is also known as a marker of osteoblastic differentiation. A monoclonal primary antibody against osteopontin followed by fluorescently labeled secondary antibody was tested in our studies.
Chapter 3

Preparation of PLL-g-PEG (Dextran)-Based Gradients

This Chapter introduces the poly(L-lysine)-graft-polymer (PEG or Dextran)-based chemistry for metal oxide surface modification, and its biological applications thanks to its well-known anti-fouling properties. Next, it details the technique used to fabricate PLL-g-PEG surface chemical gradients—the dipping method—including the measurement of adsorption kinetics with OWLS, and the set-up of a linear-motion drive for gradual dipping of substrates. This is followed by the experimental characterization of gradient samples by VASE and FM, and also theoretical calculations based on polymer physics. Finally, the storage and sterilization conditions for fabricated PLL-g-PEG gradients are described, which are relevant for the bio-adhesion studies described in the following chapters.

3.1 PLL-g-PEG and PLL-g-Dextran

Titanium, covered with its native oxide, is widely used in orthopedic and dental implants, surgical instruments and other medical applications that demand high levels of reliable performance, due to its mechanical strength, low density, and outstanding corrosion resistance [36]. Upon contact with physiological fluids (e.g. blood), a variety of proteins from body fluids immediately adsorb onto implant surfaces. Poly(ethylene glycol) (PEG) brushes have been shown to inhibit protein adsorption [37-39], and as a result cell attachment to surfaces [40,41], due to a combination of hydration of PEG chains and steric repulsion [42,43]. Carbohydrate-based polymer chains, e.g. dextran could represent an alternative to PEG, regarding nonfouling properties [44,45].

Poly(L-lysine)-graft-poly(ethylene glycol) (PLL-g-PEG) is a graft copolymer consisting of PEG chains grafted onto a polycationic PLL backbone. Adsorption of PLL-g-PEG onto oxide surfaces represents a convenient method for functionalization of metal oxide surfaces with PEG brushes [46-48]. The attachment of the polymer is based on the adsorption of the positively charged PLL backbone onto the negatively charged metal oxide surface in a physiological buffer solution via electrostatic
interactions. Previous characterization by XPS and IR has suggested that the PLL backbone binds to the metal oxide surface and the dense PEG chains extend away from the surface [46], as shown in Fig. 3.1 (c). Similar to PLL-g-PEG, PLL-g-dextran copolymers have been shown to spontaneously adsorb onto metal oxide surfaces, and to be able to greatly reduce the non-specific adsorption of proteins [49]. It was demonstrated that the chain length and the grafting ratio of the polymer both have a great influence on its anti-fouling property. PLL(20)-g(3.5)-PEG(2) and PLL(20)-g(4.8)-dextran(10) are chosen for gradient fabrication, as they present optimized copolymer architectures and the adlayers show the highest protein resistance (∼2 ng/cm²) [47, 49].

![Chemical structure](image.png)

**Fig. 3.1** Schematic illustration of (a) chemical structure of PLL-g-PEG [46]; (b) chemical structure of PLL-g-Dextran [49]; and (c) PLL-g-PEG adlayer on metal oxide surface in aqueous solutions. The positively charged PLL backbone attaches to the negatively charged metal oxide layer via electrostatic interactions. The grafted PEG chains are hydrated (represented by the H₂O molecules between the PEG chains) and
extend into the aqueous environment [46].

### 3.2 Adsorption Kinetics

As the technique for gradients preparation is based on a dipping process, the adsorption kinetics need to be determined in advance. Most commonly, the adsorption kinetics can be described by Langmuir adsorption model, such as for simple alkanethiol adsorption on gold surfaces. It is a simple, semi-empirical isotherm derived from a proposed kinetic mechanism.

\[
\theta(t) = 1 - e^{-ck_{ad}t}
\]

Eq. (3.1)

where \(c\) is the concentration of the adsorbate solution, and \(k_{ad}\) the rate constant of adsorption.

There are three assumptions within this model. (i) All binding sites should be equal, assuming the surface is homogeneous. For a flat TiO\(_2\) surface, this is almost the case. However, it is not ideally flat, but of nm roughness, and may contain some defects; (ii) At maximum adsorption, only a monolayer of adsorbate molecules is formed (\(\theta \leq 1\)). According to the previous studies [46, 49], this assumption applies to PLL-g-PEG (and PLL-g-dextran) systems; (iii) No interaction takes place between adsorbed molecules. This may be true in the very beginning of the adsorption process. However, it is mostly unlikely for higher coverage, considering the interactions between polymer chains during the change of conformation. The Himmelhaus group has investigated the adsorption of alkanethiol-terminated PEG 2000 onto gold surfaces via ellipsometry, XPS and in situ second harmonic generation, and compared it to that of C\(_{16}\)SH. The result shows that the kinetics of the polymer film formation clearly deviate from Langmuir kinetics, and their observation suggested that the rate-determining step for adsorption into the ordered high-coverage phase is governed by a conformational transition of the grafted PEG chains from amorphous coils to a brush morphology. As the surface density of PEG chains increases, the brush structure will be energetically favored due to the increase in van de Waals forces among the polymer chains and the alkyl spacer. In addition, by changing PEG chain conformation, adsorption sites that were hindered become accessible for further adsorption [62].
There are a variety of in situ or ex situ experimental methods that have been employed for the study of polymer adsorption at liquid/solid interfaces, such as ellipsometry [50], surface plasmon resonance [51], internal reflection interferometry [52], dynamic light scattering [53], surface forces apparatus [54] and so on. Among them, OWLS is one kind of in situ, real-time monitoring technique that possesses high sensitivity, making it appropriate for high-accuracy, high-efficiency acquisition of PLL-g-PEG (or PLL-g-dextran) adsorption kinetics on TiO₂-coated waveguides. The adsorption kinetics can be influenced by the concentration of adsorbate, temperature and solvent. Morgenthaler et al used OWLS to monitor the adsorption of PLL-g-PEG [23], establishing the adsorption kinetics of PLL(20)-g(3.5)-PEG(2) from a 0.02 mg/mL solution in HEPES 2 buffer in ca. 17 minutes to reach a plateau at room temperature (Fig. 3.2 a). Thus, the dipping program can be derived for a 2 cm-long sample (Fig. 3.2 b).

![Fig. 3.2](image.png)

**Fig. 3.2** (a) Adsorption kinetics of 0.02 mg/mL PLL(20)-g(3.5)-PEG(2) in HEPES 2 measured by OWLS; (b) The program of the dipping process for a 2 cm-long gradient sample.
Similarly, adsorption kinetics were obtained for 0.015 mg/mL PLL(20)-g(4.8)-dextran(10) in HEPES 1 measured by OWLS (Fig. 3.3 (a)). The dipping program was generated from it (Fig. 3.3 (b)) and then confirmed by VASE measurements of three PLL-g-dextran gradients ($R^2=0.9961$), shown in Fig. 3.3 (c).
The subsequent dipping process, the set-up of the nonlinear motion drive, and the characterization of generated gradients are described in detail in the next section, taking only PLL-g-PEG gradients as examples, as they were then subjected to systematic biological studies.

3.3 Preparation & Characterization

3.3.1 Experimental

Substrate Cleaning

TiO$_2$ thin films (15 nm) were deposited on silicon wafers (Si-Mat Silicon Materials, Germany) by means of reactive magnetron sputtering (PSI, Villigen, Switzerland) and were then diced into pieces of 25 mm x 10 mm size by a sawing machine (POWATEC GMBH, Hünenberg, Switzerland). Prior to use, the substrates were cleaned by the following protocol: (i) 2x10 min sonication in toluene, (ii) 2x10 min sonication in 2-propanol and (iii) 2 min oxygen-plasma cleaning in a plasma cleaner/sterilizer PDC–32G instrument (Harrick, Ossining, NY, USA) immediately before gradient preparation.

Preparation & Set-up

The method to fabricate surface-gradient samples was performed as described by Morgenthaler et al. [25]. Briefly, cleaned TiO$_2$ substrates were gradually dipped into 0.02 mg/mL PLL(20)-g(3.5)-PEG(2) (SuSoS AG, Dübendorf, Switzerland) in HEPES 2 solution (consisting of 10 mM 4-(2-hydroxyethyl)piperazine-1-ethane-sulfonic acid and 150 mM NaCl, pH 7.4, both from MicroSelect, Fluka Chemie GmbH, Switzerland) controlled by a programmed nonlinear motion drive (OWIS Staufen, Germany). This set-up (shown in Fig. 3.4) allows the creation of a linear gradient of PLL-g-PEG over 20 mm length after a total immersion time of 17 minutes. The device was configured to prepare 3 samples at a time by dipping them into the polymer solution in parallel.
Characterization

The thicknesses of the resulting polymer gradients were characterized by variable-angle spectroscopic ellipsometry (VASE, M-2000F, L.O.T. Oriel GmbH, Germany) equipped with a focusing probe. Measurements were conducted under ambient conditions at three angles of incidence (65°, 70°, and 75°) in the spectral range of 370–1000 nm. Spectroscopic scans were taken before and after PLL-g-PEG adsorption every 3 mm along the gradient (x-axis direction) (see Fig. 3.5). Measurements were fitted with the WVASE32 analysis software using a multilayer model for a titanium dioxide layer on silicon and a polymer adlayer. The $n$ and $k$ values for the titanium dioxide layer were fitted, and the PLL-g-PEG adlayer thickness determined by a Cauchy model ($A_n=1.45$, $B_n=0.01$, and $C_n=0$). Three gradient samples were measured and analyzed. Triplicate measurements were also carried out on each gradient sample at positions of the same x index and three different y indices (centre and ±1 mm away from the centre) to sample the homogeneity of the gradient in the y-axis direction.

Fluorescence imaging of gradient samples was achieved by means of TRITC (ca. 3%)-labeled PLL(20)-g(3.5)-PEG(2) (SuSoS AG, Dübendorf, Switzerland). Fluorescence images were collected along the gradient samples every 3 mm via an automated fluorescence microscope (Zeiss 200M with motorized excitation/emission filter wheels, LUDL), a quadband dichroic mirror (410/504/582/699) for imaging, a quadband dichroic mirror including a quadband emitter for visual inspection and shutter for epi-fluorescence illumination (Carl Zeiss AG, Germany) at a fixed exposure time of 2 s, with the filter set corresponding to the excitation wavelength ranges of TRITC. A light scratch was applied along the gradient surfaces before imaging for the convenience of focusing. The images were processed by Image J.
software, to obtain the gray values, which were normalized and compared. As references, uniform samples of the two extreme end-compositions were also prepared by immersion of TiO$_2$ samples (1 cm x 1cm) into 0.02 mg/mL PLL(20)-g(3.5)-PEG(2)/TRITC solution for 0 and 17 minutes, respectively.

Static contact-angle measurements of the generated gradients were carried out with a NRL contact-angle goniometer (model 100-00-230, Ramé Hart Inc., NJ, USA) by placing a 4 µL water droplet onto the substrates.

### 3.3.2 Results & Discussion

#### 3.3.2.1 Experimental characterization of PLL-g-PEG gradients

VASE characterization at different positions along the gradients is shown in Fig. 3.5 and the comparison with fluorescence intensity measurement in Fig. 3.6. The results from both characterization techniques showed a linear change of PLL-g-PEG surface coverage along the gradient samples. The maximum surface coverage, which was measured as 0.8 ± 0.04 nm by VASE, corresponds to immersion in 0.02 mg/mL PLL-g-PEG solution in HEPES II buffer for 17 minutes. The surface coverage continuously decreases following the gradient x-axis towards shorter dipping times until at the end that was only exposed to the solution for a very short time (several seconds), the surface is only sparsely covered with PLL-g-PEG with a dry thickness of 0.5 ± 0.05 nm. The range of the PLL-g-PEG surface coverage on the gradient samples corresponds to a polymer PEG conformation changing from the brush regime at one end to the mushroom regime at the other end, as indicated by the value of L/2R$_g$—demonstrated in the calculation below. The homogeneity of gradient samples along the y direction (i.e. perpendicular to the gradient axis) was carefully examined by VASE measurements every 1 mm, and yielded a standard deviation of less than ± 0.2 nm in thickness.

The contact angle along the entire gradient surface was essentially constant: from 28±2° at one end (∼100 % PLL-g-PEG) to 24±2 ° at the other end (bare TiO$_2$). Hydrophilic polymer brushes are known to exhibit a non-zero water contact angle [55]. The influence of change of surface wettability can be excluded in these experiments.
Fig. 3.5 Homogeneity test of three PLL-g-PEG gradient samples measured by VASE (spl. 1, 2, 3). These three samples were dipped gradually into the polymer solution at the same time. The upper sketch presents the way in which different positions on gradient samples were characterized by VASE. The conformations of PEG chains along the gradient are indicated by the value of L/2R_g.

Fig. 3.6 Normalized fluorescence intensity of TRITC-labeled PLL-g-PEG along gradient sample measured by fluorescence microscopy compared with that measured by VASE.
3.3.2.2 Theoretical Calculations

Projected monomer (EG) density

According to Pasche and coworkers [47], protein adsorption behavior is highly correlated with the projected monomer (ethylene glycol, EG) density. For this reason the PEG adlayer thicknesses along the gradient samples are converted to an EG density, in order to be able to compare different samples for protein adsorption or cell-adhesion behavior. The calculation of the EG density is given by:

\[
\begin{align*}
    n_{\text{PEG}} &= \frac{N_A m_{\text{pol}}}{M_{\text{Lys}} + M_{\text{PEG}}} \quad \text{Eq. (3.2)} \\
    n_{\text{EG}} &= n_{\text{PEG}} \frac{M_{\text{PEG}}}{M_{\text{EG}}} \quad \text{Eq. (3.3)}
\end{align*}
\]

where \( n_{\text{PEG}} \) and \( n_{\text{EG}} \) represent the surface density of PEG and EG monomers, while \( M_{\text{Lys}} \), \( M_{\text{PEG}} \) and \( M_{\text{EG}} \) are the molecular weights of the lysine monomer, PEG and EG monomers. \( g \) denotes the grafting ratio of PEG, which is 3.8 in all experiments. \( m_{\text{pol}} \) represents the mass of the polymer adlayer per unit area, the value of which can be obtained from optical waveguide lightmode spectroscopy (OWLS) measurements. \( N_A \) denotes Avogadro’s number. The \( n_{\text{EG}} \) at one end of the gradient, which was produced by an immersion time of 17 minutes, can be calculated from the formulae above. The value of \( m_{\text{pol}} \) is obtained from the literature and was measured by OWLS. As \( n_{\text{EG}} \) is proportional to \( m_{\text{pol}} \), which is proportional to the dry thickness of the polymer adlayer measured by VASE, the values of PEG and EG monomer density of different positions along the gradient samples can be calculated in the following way:

\[
\begin{align*}
    n_{\text{PEG}}' &= (m_{\text{pol}}'/m_{\text{pol}}) n_{\text{PEG}} = (h_{\text{pol}}'/h_{\text{pol}}) n_{\text{PEG}} \quad \text{Eq. (3.4)} \\
    n_{\text{EG}}' &= (m_{\text{pol}}'/m_{\text{pol}}) n_{\text{EG}} = (h_{\text{pol}}'/h_{\text{pol}}) n_{\text{EG}} \quad \text{Eq. (3.5)}
\end{align*}
\]

where \( m_{\text{pol}} \), \( h_{\text{pol}} \), \( n_{\text{PEG}} \) and \( n_{\text{EG}} \) represent, respectively, the mass of polymer adlayer determined by OWLS, the ‘thickness’ of the adlayer dry mass measured by VASE, and PEG and EG surface densities after immersion for 17 minutes, while \( m_{\text{pol}}' \), \( h_{\text{pol}}' \), \( n_{\text{PEG}}' \) and \( n_{\text{EG}}' \) refer to those values at different positions along the gradient samples.

L/2R_g

As the surface coverage of PLL-g-PEG along the gradient samples decreases, the conformation of the PEG side chains under aqueous solutions is expected to change gradually from the “brush” regime to the “mushroom” regime (Figure 1). It has been reported that a change in resistance to protein adsorption occurs around the transition
between these two regimes [56, 47]. Thus, the value of L/2Rg is an indication of the polymer conformation, where L refers to the mean distance between the PEG chains and Rg the radius of gyration of the free chains in aqueous solutions. When L/2Rg > 1, the PEG chains are in the random-coiled conformation (“mushroom” regime), and when L/2Rg ≤ 1, there is a tendency for the PEG chains to stretch out (“brush” regime).

Assuming a hexagonal close-packed arrangement of PEG chains, the transition occurs when

\[ L = 2R_g = \left( \frac{2}{\sqrt{3}n_{PEG}} \right)^{0.5} \]  

Eq. (3.6)

The radius of gyration (Rg) of the PEG chains was estimated with an empirical formula based on static light-scattering measurements [57, 47], which yields a value of 1.6 nm.

\[ R_g = 0.181A^{0.5} \text{ (nm)} \]  

Eq. (3.7)

where \( N \) is the number of the EG repeat units. From the above formulae (3), (5) and (6), the critical value of n_{PEG} distinguishing the two regimes is around 6.4/nm². Therefore, along the gradient samples the PEG chain conformation remains in the mushroom regime when n\_\text{EG} < 6.4/nm², and undergoes transformation into a brush state when n\_\text{EG} ≥ 6.4/nm².

\( S_{\text{TiO}_2} \)

\( S_{\text{TiO}_2} \) is the fraction of sample surface area that is not covered by PEG chains per unit area, meaning the fraction of the surface that exposes bare TiO₂.

When L > 2Rg, the conformation of a PEG chain is in the mushroom regime, which is considered to correspond roughly to that of the free polymer chains in solution. Thus, the value of Rg of a PEG chain in aqueous solution was used to approximate that of the grafted PEG side chain in the mushroom regime on the sample surface. In this case, the exposed TiO₂ area within the unit cell, \( A_{\text{TiO}_2} \), is simply the difference between the projected area of a PEG chain and the area of the unit cell:

\[ A_{\text{TiO}_2} = \frac{\sqrt{3}}{4}L^2 - \frac{1}{2}\pi R_g^2 \]  

Eq. (3.8)
Thus \( A_{\text{TiO}_2} = \frac{1}{2} \pi \left( \frac{1}{n_{\text{PEG}}} - \pi R_g^2 \right) \) \hspace{1cm} \text{Eq. (3.9)}

And dividing through by \( L^2 \) yields

\[ S_{\text{TiO}_2} = 1 - n_{\text{PEG}} \pi R_g^2 \] \hspace{1cm} \text{Eq. (3.10)}

When \( L/2R_g \leq 1 \), the PEG chain on the sample surface tends to gradually elongate. Therefore, in this regime, we assume a model where the radius of PEG on sample surfaces (\( R' \)) gradually becomes smaller than \( R_g \) as \( L \) becomes smaller, while the PEG chains remain in a hexagonal close-packed arrangement. In this case, the exposed substrate area \( (A_{\text{TiO}_2}) \) is effectively zero (see Fig. 3.7).

\[ \text{Fig. 3.7} \quad \text{A schematic drawing of the top view of PEG chain arrangement on the gradient surfaces in the brush regime (} L/2R_g \leq 1 \text{) and in the mushroom regime (} L/2R_g > 1 \text{).} \]

\[ \text{Fig. 3.8} \quad \text{A cartoon of the PLL-g-PEG surface gradient samples on TiO}_2, \text{ illustrating the conformations of PEG chains along the sample, ranging from the mushroom to the brush regime.} \]
3.4 Storage and Sterilization Conditions

3.4.1 Storage conditions

In case they cannot be immediately used for further applications after preparation and also for transportation purpose, the storage conditions of PLL-g-PEG gradients need to be explored. The medium, temperature, and time for storage should be taken into consideration, regarding (i) the stability of PEG, as it is known to have a tendency to degrade and eventually leads to chain scission and poor nonfouling property, which can be initiated by heat, light, etc. and accelerated by oxygen [58]; (ii) the stability of adlayer; PLL-g-PEG coating is of the purely electrostatic nature of adhesion. Therefore, this copolymer can desorb at too low or high pH, or at too high ionic strength as a consequence of screening of the electrostatic interaction; (iii) the stability of the surface-density gradient. As in bulk diffusion, the existence of the surface-density gradient may lead to the surface diffusion of adsorbed molecules from the high-concentration end to the low-concentration end, which is typically a thermal activated process with increasing rate upon increasing temperature. To meet these three requirements, it can be assumed that an aqueous solution of low ionic-strength, moderate pH at a dark, sealed, and cold environment would be the appropriate storage condition for PLL-g-PEG gradient samples.

It has been reported that a homogeneous PLL-g-PEG coating is stable at 4°C in 10mM HEPES buffer up to a few weeks [47]. Therefore, in our studies, the period and temperature for storage was examined only with respect to surface-diffusion effects. The thickness change of three gradient samples at 4 °C in MilliQ water was monitored by VASE measurement, at day 0,1,3,7 after sample preparation (shown in Fig. 3.9, from Zeng’s work of semester project). Immediately after fabrication, the thicknesses of adlayers at different positions along gradients were measured, then the samples were kept in MilliQ water in sealed cell-well plates and maintained at 4 °C in the refrigerator. After specific numbers of days, the samples were removed from the water, dried under N₂, and measured with VASE. Each time after measurements, the samples were rinsed with MilliQ water and then immersed in fresh MilliQ water and kept at 4 °C until the next time for a measurement.

The trend of all gradients before and after storage in Milli-Q water at 4°C for different times is almost the same, without a statistically significant difference. Even up to one week, no obvious decrease in thickness at the beginning or increase in the end of gradient samples was observed. In contrast, even after 1 day at 37°C in water, the diffusion effect was already detected by VASE measurements (see Fig.3.10, from
Zeng’s work of semester project). This result can be attributed to the very low diffusion rate of PLL-g-PEG on the surface at 4°C. It can be concluded that 4°C in Milli-Q water for up to 7 days is an appropriate storage condition for PLL-g-PEG gradients.

**Fig. 3.9** Influence of storage time for PLL-g-PEG gradients in water at 4°C measured by VASE.

**Fig. 3.10** Influence of storage temperature at 37°C for PLL-g-PEG gradients in water measured by VASE.
3.4.2 Sterilization/Disinfection Conditions

As the gradient samples will be applied in eukaryotic cell adhesion and microbial adhesion studies later, where cell survival and growth are sensitive to the presence of microorganisms and their colonies, it is necessary to sterilize the samples prior to use. Sterilization can be achieved by various techniques, including physical, chemical and physiochemical means, summarized as below in Fig. 3.11 [60]. To be effective, sterilization requires time, contact, temperature and, with steam sterilization, high pressure.

The choice of the sterilization/disinfection method for certain material depends not only on the type and amount of microorganism present, but also on the properties of the material in use. More details of sterilization/disinfection methods can be found in [61]. Due to the sensitivity of PLL-g-PEG, as described in section 3.4.1, harsh conditions such as autoclave, UV, and plasma are not applicable. Therefore, immersion in 70% EtOH for 20 minutes could be an appropriate candidate. To verify that this sterilization method does not change the surface modification, VASE measurements were carried out and results were compared before and after the sterilization step, and no statistical difference was found along the entire gradient samples (shown in Fig. 3.12). Therefore, for all the experiments carried out for eukaryotic cell studies in chapter 5, immediately prior to use, the gradient samples were subjected to 70% EtOH for 20 minutes, and then rinsed thoroughly with sterile water three times to remove any trace of EtOH.

![Fig. 3.11 Different methods for sterilization/disinfection](image-url)
Fig. 3.12  Sterilization condition verified with VASE measurements.
This chapter firstly provides general background knowledge of protein adsorption onto solid surfaces, focusing on the driving forces for protein adsorption and the factors influencing protein adsorption with respect to (physico-)chemical properties of both proteins and materials surfaces. It then describes the first application of PLL-g-PEG gradients in protein-adsorption studies. Adsorption of different proteins, including albumin (Ab), fibrinogen (Fgn), fibronectin (Fn) and serum, was studied using ellipsometry and fluorescence microscopy to obtain quantitative results. A competitive adsorption study was carried out with a mixed Fgn and Ab solution.

4.1 Background to Protein Adsorption

Proteins are the most abundant organic compounds found in living organisms, and thus they occupy an important position in bio-related fields of research. The study of protein adsorption at liquid/solid interfaces is not only of great interest to fundamental scientific research, but also of extraordinary value to biomedical, biochemical and biotechnology applications. In some cases, i.e. biosensors, non-specific protein adsorption needs to be minimized to reduce background signals [63]. While in other fields, e.g. implant-related biomedical applications, extracellular matrix (ECM) protein adsorption is crucial, as they are known to mediate cell adhesion, spreading and other processes, which may ultimately influence the success of implanted devices [64].

Protein adsorption can occur within seconds after contact. It is an extremely complex process, with many different steps involved, as illustrated in Fig. 4.1 [65]. In step (1), proteins are transported towards solid surfaces by diffusion and convection. Step (2) establishes the attachment of proteins on the solid surface by interactions at the interface. Step (3) involves conformation/orientation change of the adsorbed proteins, which can occur immediately upon adsorption or slowly over time. Proteins are not rigid bodies—they may orient and spread in such a way that the maximum
contact with the solid surface can be achieved, or in a way that more molecules can be adsorbed as the surface density increases with time. Furthermore, they may also have a tendency to rearrange their structures to optimize the interfacial interactions. Step (4) corresponds to detachment of proteins from the solid surface. Usually protein adsorption is not reversible or only partially reversible, since they attach to the solid surface via multiple binding sites. In step (5), proteins are transported away from the surface after desorption. The desorbed proteins can retain their altered structure or recover their native conformation, and could adsorb again. All these steps mentioned may vary in time scale, and each of step 1-3 could be the rate-determining step for the entire adsorption process, depending on the system conditions.

Fig. 4.1 Schematic drawing of protein adsorption process from aqueous solutions onto solid surfaces [65]. Step (1) transport of proteins; step (2) attachment of proteins; step (3) conformation change of proteins; step (4) desorption of proteins; and step (5) transport away of proteins. (/) denotes hydrophobic groups. (+) and (-) indicate the positively and negatively charged groups of proteins and on solid surfaces.

Protein adsorption is driven by various interfacial forces. In the current scientific view, it can be modeled by the extended Derjaguin-Landau-Verwey-Overbeek (XDLVO) theory [66], including the classic DLVO forces often applied to colloid stability in solution, comprising a combination of Van der Waals attraction and electrostatic repulsion forces, and non-DLVO forces, e.g. hydration forces, hydrophobic interactions, depletion forces. Depending on the conditions of the system under study, the dominant driving forces can be different. From a thermodynamic perspective, adsorption is a spontaneous reaction, if
\[ \Delta_{ads} G = \Delta_{ads} H - T \Delta_{ads} S < 0 \]  

Eq. (4.1)

where G, H, S, T represent the Gibbs free energy, enthalpy, entropy and temperature, respectively, and \( \Delta_{ads} \) denotes the change after adsorption process. In many cases, the surface-induced conformation changes of proteins lead to an entropy gain, and thus can greatly contribute to the driving force for adsorption.

Important parameters for protein adsorption include the structures and properties of proteins, properties of solid surfaces, pH and ionic strength of solution, and temperature [67]. These factors can influence both the amount and conformation/orientation of adsorbed proteins.

Regarding protein properties, the complexity arises from the fact that each type of protein has a unique function and nature. Characteristics such as size, shape, charge and internal stability, can play an important role in the outcome of adsorption. A good example with respect to the role of shape is shown in Fig.4.2 [68], where the rod-like fibrinogen undergoes an orientation change from “side on” type to “end on” type adsorption, as the amount of adsorbed protein increases, while the conformation of a globular protein BSA is only slightly distorted upon adsorption without rearrangement of orientation. There are also some general observations regarding the internal stability of protein structures [67] [69]. Those protein molecules of high internal stability are termed “hard proteins”, such as lysozyme, ribonuclease, etc. In contrast, the so-called “soft proteins” have a low internal stability, for example, albumin, immunoglobulin, fibrinogen, etc. Generally, “hard proteins” adsorb on hydrophobic surfaces under a wide range of conditions, whereas on hydrophilic surfaces they adsorb only in case of strong electrostatic attraction. “Soft proteins” show a higher affinity for all surfaces, and for “soft proteins”, denaturing on solid surfaces is much more likely than for “hard proteins”.

In order to understand protein adsorption, it is also quite important to bear in mind the influence of properties of the adsorbent surface. As was previously reported, both surface topography and chemistry can have distinct effects on protein adsorption [70-71]. Many attempts have been made to characterize the influence of surface chemistry through the use of different materials and methods. Different materials surfaces, ranging from bare metal, metal oxide and ceramic surfaces to well-defined SAM systems (especially the Au/thiol system) and polymer systems, have been extensively employed as model surfaces, often involving homogeneous coatings or even surface-chemical gradients. These systems vary in surface charge, wettability, nonfouling
capability and other (physio-)chemical properties. Characterization of protein adsorption on these systems has typically been performed by measuring the adsorption isotherm, adsorption and desorption kinetics by OWLS, SPR, QCM-D; the amount of adsorbed proteins by XPS, VASE, ELISA, FM; conformation/orientation of adsorbed proteins by FT-IR, CD, AFM, ELISA; distribution of adsorbed proteins by AFM, SEM, FM and other physical parameters related to the adsorbed protein layer, such as layer thickness and refractive index by VASE [72]. For example, Scotchford et al. investigated fibronectin (Fn) and albumin (Ab) adsorption on SAMs on gold with different termini through fluorescence immunocytochemistry, and results showed that the amount of Fn adsorption followed the sequence COOH>OH>CH₃, whereas for albumin, it was OH>COOH>CH₃[73].

![Fig. 4.2 Adsorption of (a) a globular protein (e.g. BSA), whose conformation can become slightly distorted on surfaces, and (b) a rod-like protein (i.e. fibrinogen), which undergoes a multistage conformation/orientation change where initially it adsorbs in a “side-on” orientation and later in an “end-on” orientation due to the increase of surface density and increasing protein-protein interactions [68].](image)

In many cases, it is unlikely that only one type of protein is present in solution. This situation is normal for body fluids, blood, containing more than 150 types of proteins, serves as a good example. In such a multi-protein adsorption system, differences between concentrations of each protein, the interaction of each protein with adsorbent surfaces, and even the lateral protein–protein interactions, determine the outcome of the complex adsorption process [72]. Often, the competitive nature of the protein–surface interactions results in transient dominance of one/certain protein(s) over the others. This phenomenon was first observed and demonstrated by Leo Vroman in 1960s [74], who suggested that blood-protein adsorption involved a hierarchical series of collision, adsorption and exchange processes. It was further confirmed later by Brash and Hove [75] and others, and came to be known as the “Vroman effect”, that is, small proteins of high concentration may dominate the adsorption sites at early stage of the competitive adsorption process, whereas later larger and less abundant proteins may replace some/all of the already adsorbed
proteins. Although the detailed molecular mechanisms of the Vroman effect are still not fully understood, the outcome of the competitive adsorption with respect to the influence of adsorbent surface properties has been experimentally confirmed through different methods and techniques. For instance, the use of a surface wettability gradient combined with the TIRFM technique by Elwing et al. revealed the undeniable effect of surface hydrophobicity/hydrophilicity on dynamic replacement of fibrinogen by other serum proteins [76].

As surface gradients present a more convenient way for systematic studies, a number of reports investigating protein adsorption and even further cellular behavior utilizing surface chemical gradients have appeared. For examples, Lee et al. created a wettability gradient by treating polyethylene in a corona discharge of gradually changing power, and subsequent protein-adsorption experiments characterized by XPS and ATR illustrated that albumin adsorption increases as surface hydrophobicity increases [77]. Mei et al. produced a poly(HEMA) gradient, by means of in situ controlled radical polymerization, to modify fibronectin adsorption and thus to tune cellular adhesion [78]. However, the substrates (e.g. silicon or gold) often had little relevance to implant materials, but rather were used for their ease of chemical modification. We have employed PLL-g-PEG gradients on titanium oxide surfaces using the simple and reproducible method described in the last chapter. By controlling the density of surface-exposed/linked PEG chains, it is possible to modulate the amount of protein attachment and thereby to influence the extent to which cells attach. Depending on the surface coverage of PEG, the PEG chain changes its conformation from the mushroom to the brush regime, which could show different protein-adsorption characteristics. The proteins under study are relevant to implants: albumin (Ab), fibrinogen (Fgn), fibronectin (Fn) and serum. The other conditions that could influence protein adsorption, such as pH and ionic strength of solution and temperature, were kept constant at physiological conditions (pH=7.4, 150 mM, 37°C).

4.2 Single-protein adsorption

4.2.1 Experimental

Protein Adsorption: PLL-g-PEG gradient samples were incubated in 1 mg/mL Alexa 488-labeled BSA (Invitrogen, Basel, Switzerland), or 50 µg/mL Alexa 546-labeled Fgn (Invitrogen, Basel, Switzerland), or 30 µg/mL Fn (obtained in lab) at 37°C for 30 minutes, or 10% serum (FBS South American H/I, Gibco, Invitrogen, Basel,
Switzerland) for 30 minutes, 1 hour and 4 hours, followed by rinsing with HEPES 2 and abundant Milli-Q water, and then dried with N₂. The experiments were carried out in triplicate for each incubation time.

BSA and Fgn adsorptions were characterized by two techniques: (i) the thickness of the adsorbed protein layer was determined every 3 mm along the gradient samples by VASE, by subtracting the initial polymer adlayer thickness from the total adlayer thickness after exposure to the protein solution. The optical constants of the polymer and the protein layers were fixed, using the Cauchy model as described in the last chapter. (ii) Fluorescence intensity of the protein adlayer was measured by taking fluorescence images every 3 mm along gradient samples via an automated fluorescence microscope (Zeiss 200M with motorized excitation/emission filter wheels, LUDL), a quadband dichroic mirror (410/504/582/699) for imaging, a quadband dichroic mirror including a quadband emitter for visual inspection and shutter for epi-fluorescence illumination (Carl Zeiss AG, Germany) at a fixed exposure time of 2 s. A light scratch was applied along the gradient surfaces before imaging for the convenience of focusing. The images were then processed by ImageJ software to obtain grayscale values and then the gray value of the bare substrate was subtracted. The data obtained from VASE and fluorescence intensity measurements were normalized and compared.

For Fn and serum-protein adsorption, only VASE measurements were applied to obtain the adsorption thickness and mass.

4.2.2 Results

Albumin and fibrinogen are two of the most abundant serum proteins, and are also among the most significant proteins that adsorb on biomaterial surfaces. The comparison of the results from the two techniques is plotted in Fig. 4.3. For both BSA and Fgn adsorption, the amount of adsorbed proteins gradually increases as the polymer density (expressed as projected surface monomer density of EG) decreases on the surfaces. In agreement with previous literature [25], it is also observed that significant inhibition of Fgn adsorption (>95%) requires a higher EG density (12.8±0.6/nm²) compared to that necessary to inhibit BSA adsorption (8.3±0.8/nm²). The shapes of the curves are slightly different—while BSA adsorption reveals a more exponential-like curve as a function of EG density, Fgn adsorption shows a more linear curve at lower EG densities. Fn is one of the most important ECM proteins. Its adsorption behavior on PEG gradient samples reveals similar as that of Fgn, and it
also requires a higher EG density (13.1±0.9/nm$^2$) to inhibit adsorption significantly (>95%) (shown in Fig. 4.4).

The total amount of protein adsorption from 10% serum was measured shows a similar general trend on the PLL-g-PEG gradient samples with a slight increase at every position as incubation time increases, plotted in Fig. 4.5.

The surface mass density for single-protein adsorption was calculated from the dry thickness measured by VASE, also shown in Fig 3. Here, the Stenberg and Nygren formula [79] was applied to give:

$$\Gamma \approx K \cdot d_{\text{dry protein}}$$

Eq. (4.2)

Where $K \approx 1.2 \times 10^6$ ng/mm$^3$, and $d_{\text{dry protein}}$ is the dry thickness value of adsorbed protein measured by VASE.
**Fig. 4.3** Comparison of VASE and fluorescence intensity measurements of single protein adsorption along PLL-g-PEG gradient samples. (a) Alexa 488-labeled BSA adsorption in 0.5 hr; and (b) Alexa 546-labeled Fgn adsorption in 0.5 hr.

![Graph](image1)

**Fig. 4.4** Adsorption of Fn along PLL-g-PEG gradient samples in 0.5 hr measured by VASE.

![Graph](image2)

**Fig. 4.5** Adsorption of serum proteins in 0.5 hr, 1hr and 4hr along PLL-g-PEG gradient samples measured by VASE.
4.2.3 Discussion

From the semi-empirical geometric model of PEG chains shown in chapter 3.3.2.2, single-protein adsorption along the PLL-g-PEG gradient can be described by a simplified model where proteins occupy the exposed TiO$_2$ area:

$$\Gamma = \Gamma_0 S_{TiO_2} \quad \text{Eq. (4.3)}$$

Where $\Gamma_0$ is the surface density of protein adsorption on bare TiO$_2$ substrates.

The comparison of the theoretical model and the experimental data by VASE is shown in Fig. 4.6 (a). Both BSA and Fgn adsorption, measured by VASE, generally display a comparable behavior, in which in the brush regime there is negligible protein adsorption, due to the hydration and steric repulsion effects of the PEG chains, while in the mushroom regime it gradually increases as EG density decreases. However, it is clear that BSA adsorption over the entire mushroom regime is overestimated by the linear model described above, and best fitted by an exponential decay function ($y=-0.0273+1.02\times\exp(-x/3.65)$, $R^2=0.9938$). This can be attributed to the influence of protein size and geometry, requiring more energy to compress PEG chains for protein adsorption if the size of the protein molecule is greater than the distance between PEG chains (L), as has been reported [56]. The effect of size was confirmed by previous studies, in which gradients of two proteins were fabricated by making use of their size differences [80]. The experimental data of Fgn exhibits a linear tendency in the mushroom regime ($y=-0.0964x+1.03$, $R^2=0.9976$), and the transition point occurs at a higher EG density than that of BSA. As Fgn (340 kDa) is a much bigger protein than BSA (67 kDa), the difference of the adsorption behavior cannot be simply attributed to either size or molecular weight, but also to the structures of the proteins, the multiple binding sites and the conformation/orientation of the protein upon adsorption.

BSA is commonly modeled as an ellipsoid of dimension 14*4*4 nm$^3$ [81]. The isoelectric point (IEP) of BSA is 4.7 and it has a uniformly distributed charge on the outer, solvent-exposed surface. Fgn, on the other hand, is a dimeric protein of 47.5*6*9 nm$^3$ size with an IEP of 5.5, modeled as a trinodule comprised of three spherical regions connected by two narrow rods. According to the current view [82], the protein consists of two sets of three non-identical polypeptide chains, which form three domains—two D domains (6.5 nm) and one E domain (5 nm). These three domains are negatively charged at physiological pH, while the two small, extended $\alpha$C domains (<1 nm, fibrinopeptides) folded by the extended $\alpha$ chain are positively
charged. Protein adsorption is generally considered to be driven by DLVO forces and other, non-DLVO forces, such as acid-base interactions and hydrophobic interactions. In our system, the hydrophobic interactions are believed to be low, since the entire gradient surface is hydrophilic. As for TiO$_2$, whose IEP is reported to lie between 4.0 to 6.2, it is negatively charged at physiological pH. Thus it was suggested that protein adsorption on oxide surfaces is partly attributable to salt bridges or electrostatic interactions [83]. For Fgn, it is quite possible that only the positively charged $\alpha$C domains are closely attached to the exposed negatively charged TiO$_2$ surface, while the other Fgn domains rise up, as has been reported on other hydrophilic, negatively charged substrates [83-85]. This might result in a much lower energy cost for Fgn to squeeze in between PEG chains and attach to the exposed TiO$_2$ surface, and therefore it can adsorb in a higher-n$_{EG}$ region than can BSA (Fig. 4.6). In other words, while for Fgn, it is simply sufficient for a certain amount of bare TiO$_2$ to be present, in order for the molecule to find an attachment point, in the case of BSA, there appears to be a requirement for a certain critical area of bare TiO$_2$. This accounts for the steeper drop-off of the BSA curve. From our experimental data, it can be concluded that not only the size of protein as described before, but also the tertiary structure, the charge distribution and other physiochemical properties (eg. multiple binding sites) may affect the protein interaction with PEG-grafted surfaces in a way that finally determines the total amount of adsorption and also the orientation/conformation of the adsorbed protein.
Fig. 4.6 (a) Comparison of theoretical and experimental protein adsorption along the PLL-g-PEG gradient; and (b) A schematic drawing of Fgn and BSA adsorption on PEG gradients, showing that the positively charged small fibrinopeptides might adhere to very small TiO$_2$ surface areas through electrostatic interactions, while BSA cannot, due to the limitations of its size and structure.

4.3 Competitive protein adsorption

4.3.1 Experimental

A protein solution containing 1 mg/mL Alexa 488-labeled BSA, or 50 µg/mL Alexa 546-labeled fibrinogen, or the mixture of these two proteins of the same final concentration as described above, were prepared and incubated with PLL-g-PEG gradient samples for 10 minutes, 30 minutes, and 4 hours, respectively. At least 3 samples were used for each condition. Fluorescence images were collected along the gradient samples every 3 mm with two filter sets corresponding to the excitation wavelength ranges of Alexa fluor 488 and 546. The images were processed by Image J to obtain the gray values, normalized and compared.

For purposes of calibration of FM intensity and comparison, VASE measurements were used for BSA and fibrinogen single-protein adsorption assays at 37°C for the time point of 30 minutes.

4.3.2 Results & Discussion

Adsorption from single-protein solution on PLL-g-PEG gradient samples was measured by two different techniques—VASE and FM. While VASE gives an absolute value of the total protein adlayer thickness, irrespective of the conformational change of proteins upon adsorption, fluorescence-intensity measurements (FM) offer a complementary, semi-quantitative yet direct determination of the number of adsorbed protein molecules. The comparison of the results from the two techniques is plotted in Fig. 4.3. It shows that the results gained from the two techniques correlate well with each other within the error bars, confirming that the detection limit, accuracy and precision of FM measurements at these experimental conditions are comparable to those of VASE. Therefore, the same experimental conditions of FM were applied to the study of competitive adsorption of protein solutions containing both albumin and fibrinogen.
In our study, the complex process of protein adsorption from plasma onto oxidized titanium surfaces was modeled as competitive adsorption from a mixture of Fgn and BSA. The concentration ratio (w/w, Fgn:BSA=1:20) was chosen according to the physiological conditions in human plasma, which contains 20-40 mg/mL albumin and 1-2 mg/mL fibrinogen. The adsorption kinetics were monitored by FM at three time points—10 minutes, 0.5 hr and 4 hr. The results were compared with those from single-protein solutions, as shown in Fig. 4.7. However, our results show that the fluorescence intensity of Alexa 488-BSA was below the detection limit (roughly below 10 ng/cm$^2$) along the whole gradient sample at 10 min, 30 min and 4 hr, while the fluorescence of Alexa546-Fgn was almost the same as that from single-protein Fgn adsorption. The same phenomena was observed upon reducing the Fgn
concentration in the mixture solution (w/w, Fgn:BSA=1:100) (data not shown). From these results, one can conclude that a preferential adsorption of Fgn occurs compared to that of BSA onto the entire gradient samples over the studied time period. Alternatively, protein replacement could have already taken place within the first 10 min.

When BSA and Fgn were used competitively, one could have expected, according to the study of single-protein adsorption and consistent with the Vroman effect [74], that the smaller protein—BSA—would diffuse and adhere to the exposed regions of the PLL-g-PEG gradient surface first, except in the higher $n_{EG}$ region, where Fgn adhesion is favored. BSA would then later be replaced by Fgn along the entire gradient. However, over the time interval we studied, which was believed to be critical for the subsequent cell adhesion, Fgn adsorption dominated over the entire gradient surface, indicating a much higher tendency for Fgn adsorption on hydrophilic TiO$_2$. It is likely that the much higher affinity leads to the very fast exchange of protein adsorption over a few minutes or even seconds, and therefore the expected influence of PEG density was not detected. The high affinity of Fgn for TiO$_2$, compared to that of BSA has also been reported previously, although not in a direct competitive assay. It was claimed that Fgn has at least twice the affinity towards titanium surfaces compared to albumin.

4.4 Conclusion

In summary, through the use of surface gradients of PLL-g-PEG fabricated by a dipping process, we have carried out a quantitative investigation of protein adsorption on titanium surfaces, including albumin, fibrinogen, fibronectin and serum. The single-protein adsorption behavior and the competitive adsorption were measured with VASE and FM. The adsorption behavior of single proteins generally correlated with semiempirical geometric models, illustrating the effect of the PEG-chain surface distribution on the inhibition of protein adsorption. Distinct differences could be observed between individual adsorbing proteins, attributable to their mode of surface attachment. The competitive adsorption of protein solutions containing albumin and fibrinogen was then investigated by FM, indicating a larger amount of fibrinogen adsorption compared with albumin adsorption (in minutes to hours) along the entire PLL-g-PEG gradient samples. The use of surface-gradient samples demonstrated the importance for protein adsorption of PEG conformation, the amount of exposed
titanium dioxide surface area (and its distribution), and the structure and chemistry of the proteins involved.
Building upon the last two chapters, in this chapter, a detailed study of tissue cell-materials interactions was carried out through the use of PLL-g-PEG gradients and protein-backfilled gradients. Firstly, adhesion assays were carried out with human foreskin fibroblasts (hFF), so as to further elucidate the underlying mechanism of cell adhesion and spreading as a function of PEG coverage and the potential involvement of integrins. Subsequently, regarding the implant-related applications, cell proliferation, viability and other aspects were investigated with hFF and RCO (rat calvarial osteoblasts) cell models.

5.1 Background to Cell Adhesion

Eukaryotic cells can attach to surfaces, extracellular matrix (ECM) and even other cells, both in vivo and in vitro. Cell adhesion plays many vital roles in numerous cellular processes. As the information from surfaces/ECM can be transmitted to the cells following attachment, activating cell-signaling pathways, attachment is key to the regulation of cell growth, differentiation, apoptosis and other important cell activities [86]. In implant-related biomedical fields, it is therefore essential to gain a better and detailed understanding of cell-adhesion behavior and the mechanisms involved.

Generally, there are two types of adhesion that can be distinguished. The first type, “non-specific adhesion”, refers to physico-chemical interactions at interfaces. Similar to protein adsorption, it can be described by the extended DLVO theories, and is mainly mediated by surface physical and chemical properties, such as surface free energy, surface charge and so on. It therefore occurs at large and intermediate separation distances between cell and the other surfaces. The other type, which is more important, called “specific adhesion”, involves a specific recognition between receptors (cell surface-adhesion molecules) and ligands, corresponding to short-range
interactions. Specific adhesion is more important, as it leads to subsequent stable adhesion and cell spreading. There are four major families of cell surface-adhesion molecules, which are immunoglobulin (Ig) superfamily cell-adhesion molecules (CAMs), integrins, cadherins, and selectins [87]. Among them, cell-matrix/material surface adhesion is mainly mediated by the integrin family. Integrins are transmembrane proteins consisting of non-covalently linked heterodimers of alpha and beta subunits. There are 15 α subunits and 8 β subunits that have been identified [88], which can be combined in various ways to form different types of integrin receptors. For example, osteoblasts express several integrins—including α1β1, α2β1, α3β1, α4β1, α5β1, α6β1, and α8β3 [89]. Depending on cell activation states, maturity and lineage, their expression of various integrins may be altered. Some ECM proteins such as fibronectin, collagen and vitronectin are the ligands to several different integrin receptors. Upon ligand binding, integrin receptors rapidly link to the actin cytoskeleton and cluster together, to form focal adhesions. A focal adhesion is a discrete complex containing structural and signaling molecules, and has the functions to mediate adhesion strengthening, migration, and cell growth and differentiation, together with growth-factor receptors [90]. Specific adhesion is typically influenced by the bio(chemical) properties of both cell and material surfaces, as well as some physical properties (i.e. surface topography).

![Fig. 5.1](image.png)

**Fig. 5.1** An illustration of integrin-mediated cell adhesion to extracellular matrices. It involves integrin binding and clustering, focal adhesion assembly, and cytoskeletal interactions. Focal adhesions are supramolecular assemblies containing structural and signaling components regulating cell functions [90].

For implants, upon contact with physiological fluids (e.g. blood), a variety of proteins (including albumin, fibrinogen, fibronectin, vitronectin) from body fluids adsorb onto implant surfaces within seconds to minutes after implantation. The type,
the amount, and the conformation/orientation of the adsorbing protein subsequently influence the overall kinetics and thermodynamics of the binding events between cells and implant surfaces [91]. Interfacial interactions are crucial in determining the success of implant devices, as, on the one hand, certain adsorbed adhesive proteins mediate the attachment and activation of platelets, macrophages, and other inflammatory cells, triggering clotting and immune responses in the host [92]; while on the other hand, the adsorbed ECM proteins assist in specific cell adhesion and spreading through integrin binding, and regulate other subsequent signaling events and cell functions. Therefore, a significant amount of experimental and theoretical research has been focused on the investigation, understanding and control of protein adsorption, cell adhesion, and the relationships between the two. For example, the Garcia group utilized different thiol SAMs to demonstrate that surface chemistry modulates Fn conformation and directs integrin binding and specificity of cell adhesion [93]. This group also studied the influence of surface-induced Fn conformation changes in cell proliferation and differentiation [94]. Choee et al. showed the influence of surface wettability on cell adhesion and growth by using a corona-treated PE gradient [95]. Mei et al. produced a poly(HEMA) gradient, by means of in situ controlled radical polymerization, to study fibronectin adsorption and the subsequent cellular adhesion behaviors [78]. These studies focussed on the adhesive mechanisms directing cell-surface interactions, and also indicated various strategies of surface-induced control of adsorbed proteins to modulate cell functions in biomedical and biotechnological applications.

To our knowledge, however, there have been no studies reported to date on cell-protein interactions with implant-relevant surfaces that have employed a gradient in resistance to protein adsorption. In the last chapter, we have employed PLL-g-PEG gradients on titanium oxide surfaces to study the adsorption behaviors of albumin, fibrinogen, fibronectin and serum proteins. In this chapter, cell adhesion, proliferation and viability were studied in detail, with respect to the influence of PLL-g-PEG surface density and the adsorbed proteins. Two relevant primary cell models were employed as test cells. Fibroblasts (human foreskin fibroblasts, hFF) are always present and in contact with implant materials following surgery, and osteoblasts (rat calvarial osteoblasts, RCO) are responsible for bone formation.
5.2 Cell Adhesion

5.2.1 Experimental

5.2.1.1 Cell Culture

Human-foreskin fibroblasts (hFF, PromoCell, Germany) were maintained in DMEM supplemented with 10 % FBS and 1 % penicillin/streptomycin (Invitrogen, Basel, Switzerland), and kept in a humidified incubator of 5% CO\textsubscript{2} balanced-air atmosphere at 37°C. hFFs between the 8th and 9th passage were used for the experiments. After reaching confluence of 70-90%, cells were harvested by 0.25 % Trypsin-EDTA (Sigma-Aldrich, Buchs, Switzerland) treatment for less than 5 minutes in the incubator, centrifuged for 5 minutes at 1000 rpm, re-suspended and diluted to the required seeding density in the medium as required. The seeding density was optimized to be 5300 cells/cm\textsuperscript{2} for the adhesion assays (0.5 to 4 hrs) so that the adhered cells would not reach 100 % confluence but were still sufficient for statistical analysis. PLL-g-PEG gradients were placed in 60 mm Petri dishes (NuncIon® cell-culture dishes, Thermo Fisher Scientific, Denmark) where hFFs were immediately seeded and then kept in the incubator at 37°C and 5% CO\textsubscript{2} for the designated period of time.

All the following cell-adhesion assays were carried out in triplicate at each time and repeated at least once.

5.2.1.2 Cell-adhesion assays

Assay conditions are summarized in Table 5.1, and Fig. 5.2.

(a) Cell adhesion in serum-free medium: hFFs were seeded and incubated on PLL-g-PEG gradient samples for 0.5 hour, 1 hour and 4 hours in serum-free DMEM.

(b) Cell adhesion in cell-culture medium with serum: hFFs were seeded and incubated on PLL-g-PEG gradient samples for 0.5 hour, 1 hour and 4 hours in cell-culture medium (DMEM containing 10% FBS).

(c) Cell adhesion in cell-culture medium pre-incubated with PLL-g-PEG gradient samples: gradient samples were firstly incubated in cell-culture medium (DMEM containing 10% FBS) for 4 hours, rinsed three times with sterile PBS and then hFFs were immediately seeded and incubated for 1 hour in serum-free DMEM.
(d) Cell adhesion on BSA-pre-incubated PLL-g-PEG gradient samples: gradient samples were first incubated with BSA (1 mg/mL) for 0.5 hour, rinsed three times with sterile PBS and then hFFs were immediately seeded and incubated with samples for 1 hour in serum-free DMEM.

(e) Cell adhesion on Fgn-pre-incubated PLL-g-PEG gradient samples: gradient samples were first incubated with fibrinogen (50 µg/mL) for 0.5 hour, rinsed three times with sterile PBS and then immediately hFFs were seeded and incubated with samples for 1 hour in DMEM without serum.

(f) Adhesion-blocking experiments were carried out to determine the extent of specific integrin binding: hFFs were harvested and incubated with 500 µg/mL cyclic RGD (cyclo-RGDFV, Calbiochem, USA) in PBS for 30 minutes at 37°C and 5% CO₂, respectively. The cells were then centrifuged at 1000 rpm for 3 minutes, and non-bound cyclic RGD solution was carefully removed. After suspension in 1 mL sterile PBS and further centrifugation at 1000 rpm for 3 minutes to remove the residual RGD, the cells were suspended and diluted in serum-free DMEM. The cells were then immediately seeded and incubated on Fgn-pre-incubated PLL-g-PEG gradient samples.

(g) Cell adhesion on Fgn-pre-incubated PLL-g-PEG gradient samples: gradient samples were first incubated with fibronectin (30 µg/mL) for 0.5 hour, rinsed three times with sterile PBS and then immediately hFFs were seeded and incubated with samples for 1 hour in DMEM without serum.

(h) Adhesion-blocking experiments were carried out for Fn-pre-incubated PLL-g-PEG gradient samples in the similar way as in f): hFFs were harvested and incubated with 500 µg/mL cyclic RGD in PBS for 30 minutes at 37°C and 5% CO₂, respectively. The cells were then centrifuged at 1000 rpm for 3 minutes, and non-bound cyclic RGD solution was carefully removed. After suspension in 1 mL sterile PBS and further centrifugation at 1000 rpm for 3 minutes to remove the residual RGD, the cells were suspended and diluted in serum-free DMEM. The cells were then immediately seeded and incubated on Fn-pre-incubated PLL-g-PEG gradient samples.
Table 5.1 Different conditions used for cell-adhesion assays.

<table>
<thead>
<tr>
<th>No. of assay</th>
<th>gradient pre-exposure</th>
<th>hFF pre-incubation</th>
<th>hFF incubation solution</th>
<th>hFF incubation time</th>
</tr>
</thead>
<tbody>
<tr>
<td>a</td>
<td>no</td>
<td>no</td>
<td>Serum-free DMEM</td>
<td>1hr</td>
</tr>
<tr>
<td>b</td>
<td>no</td>
<td>no</td>
<td>DMEM+10% serum</td>
<td>0.5hr, 1hr, and 4hr</td>
</tr>
<tr>
<td>c</td>
<td>DMEM+10% serum for 4hr</td>
<td>no</td>
<td>Serum-free DMEM</td>
<td>1hr</td>
</tr>
<tr>
<td>d</td>
<td>1 mg/mL BSA for 0.5hr</td>
<td>no</td>
<td>Serum-free DMEM</td>
<td>1hr</td>
</tr>
<tr>
<td>e</td>
<td>50 µg/mL Fgn for 0.5hr</td>
<td>no</td>
<td>Serum-free DMEM</td>
<td>1hr</td>
</tr>
<tr>
<td>f</td>
<td>50 µg/mL Fgn for 0.5hr</td>
<td>cyclo-RGDfV</td>
<td>Serum-free DMEM</td>
<td>1hr</td>
</tr>
<tr>
<td>g</td>
<td>30 µg/mL Fn for 0.5hr</td>
<td>no</td>
<td>Serum-free DMEM</td>
<td>1hr</td>
</tr>
<tr>
<td>h</td>
<td>30 µg/mL Fn for 0.5hr</td>
<td>cyclo-RGDfV</td>
<td>Serum-free DMEM</td>
<td>1hr</td>
</tr>
</tbody>
</table>
Fig. 5.2 Schematic diagrams of all the cell adhesion assays carried out in the experiments. (a)-(h) correspond to the experimental descriptions in the section 5.2.1.2, respectively. (a) Cell adhesion in serum-free medium; (b) Cell adhesion in cell-culture medium containing 10% FBS; (c) Cell adhesion on PLL-g-PEG gradient sample pre-exposed to cell-culture medium containing 10% FBS; (d) Cell adhesion on BSA-pre-incubated PLL-g-PEG gradient samples; (e) Cell adhesion on Fgn-pre-incubated PLL-g-PEG gradient samples; (f) Adhesion-blocking experiments by pre-incubation of hFF with cyclic RGD before seeding on Fgn-pre-incubated PLL-g-PEG gradient samples; (g) Cell adhesion on Fn-pre-incubated PLL-g-PEG gradient samples; (h) Adhesion-blocking experiments by pre-incubation of hFF with cyclic RGD before seeding on Fn-pre-incubated PLL-g-PEG gradient samples; (i) The key to all the symbols in the diagrams. The results of condition (a), (d) were displayed in Fig. 5.4, (b), (c) in Fig. 5.5, (e), (f) in Fig. 5.6, and (g), (h) in Fig. 5.7.

5.2.1.3 Cell staining

For short-term adhesion assays, the samples were removed from the incubator and gently washed with PBS (Fluka Chemicals, Buchs, Switzerland) solution pre-warmed to 37°C (different washing methods were tested), and cells were fixed in 4 % paraformaldehyde solution (freshly prepared from a 20 % stock solution; PFA powder
was purchased from Fluka Chemicals, Buchs, Switzerland) for 15 minutes and then gently rinsed twice with PBS. The adhering cells were permeabilized in 0.5 % Triton X-100 in PBS (Fluka Chemicals, Buchs, Switzerland) for 5 minutes and then rinsed twice with PBS. They were subsequently stained with Alexa Fluor 488 phalloidin (1:100 dilution in PBS; Invitrogen, Basel Switzerland) for 30 min. After the samples were rinsed twice with PBS, the nuclei were stained with DAPI (1:1000 dilution in PBS; Invitrogen, Basel, Switzerland) for 15 min and rinsed twice with PBS. Finally the gradient samples were mounted onto glass slides for fluorescence microscopy.

5.2.1.4 Imaging and evaluation

All the fluorescence images were taken with an automated fluorescence microscope (Axio IMAGER M1m, Zeiss, Oberkochen, Germany). In order to investigate the density and morphology of the adhering cells, three images were taken at 8 positions (3 mm apart, the same as in Chapter 3.3) along the PLL-g-PEG gradient samples with a DAPI (for DAPI or Hoechst stain, both from Invitogen) or Fluor 488 (for Phalloidin) filter set, and at x10, and x20 magnification, respectively. A total of at least 6 samples were investigated for each condition. For analysis, images of cell nuclei and cell morphology were processed with ImageJ software. Comparisons between measurement series were made using nonparametric tests because variances were not homogeneous and observed values were not distributed normally, even after appropriate transformations. All mean values are shown ± SEM (standard error of the mean). The level of significance p was set at <0.05.

5.2.2 Results

5.2.2.1 Different washing protocols to remove unbound cells

Prior to fixing cells, the samples with attached cells have to be washed with buffer solution to remove the unbound cells in the suspensions. A commonly reported protocol (method (a)) is to aspirate the cell suspension, and then to immediately add fresh buffer with pipette for rinsing, and repeat once if necessary. However, for short-term adhesion experiments, even by visual inspection, it was obvious that after the rinsing step, many attached cells were also washed away, leaving some blank patches with no cells at random position on homogeneous TiO$_2$ samples. Statistical analysis of the results on bare TiO$_2$ samples also shows a large standard error of the mean (Fig. 5.3(a)). This common protocol doesn’t appear to be a reliable and repeatable way to retain the attached cells while removing the cell suspension. It may be due to two possible causes: (i) For 1hr incubation time, cells have not yet established strong
adhesion onto the surface (i.e. focal adhesion), and thus they are sensitive to the shear stress generated from the flow when rinsing with buffer; (ii) The sample surface is not completely hydrophilic, especially compared to the tissue culture plate where the samples were placed for seeding cells. Therefore, after rinsing with buffer, the sample surfaces could become easily dehydrated after the cell suspension is aspirated. After adding fresh buffer, the samples become hydrated by the flow. Thus, the attached cells could detach from sample surfaces by shear stress.

Two approaches were tested to optimize the washing step. Method (b), a layer of parafilm was placed and pressed tightly onto tissue-culture well plates, prior to seeding cells, and the washing step was the same as in the common protocol, i.e. aspiration and rinsing. As parafilm is quite hydrophobic (CA. 97±2°), aqueous solution could be still kept on top of sample surfaces after aspiration and rinsing. Method (c), samples were removed from the cell suspension vertically with tweezers (samples were always kept horizontal) so that a droplet of aqueous solution was still retained and covered the entire sample surface, and then gently immersed into fresh PBS buffer solution (only vertical movement) to wash away the rest of cell suspension, and finally transferred to PFA fixation solution.

From Fig. 5.3(a), it can be observed that both method (b) and (c) can retain significantly more attached cells compared to the common protocol (a), and that the standard errors of the mean are also lower. For method (b), occasionally the parafilm may float away if not pressed tightly enough, and also it requires an additional UV sterilization step, while method (c) generated somewhat better results regarding both attached cell number and standard error, and is more facile and straightforward. Therefore, method (c) was employed as the optimal washing protocol for all the short-term cell-adhesion experiments.

![Fig. 5.3](image)

**Fig. 5.3** Results of adhered cell number on bare TiO₂ substrates observed by fluorescence microscopy using different washing protocols. (a) refers to common
rinsing method, (b) adding parafilm onto well plates prior to seeding cells, and (c) samples were always vertically removed from one solution and then immersed vertically into another solution.

5.2.2.2 Cell adhesion on gradients backfilled with various proteins

To elucidate the underlying mechanisms, a series of cell-adhesion assays was carried out under different conditions: in serum-free medium, on BSA-pre-adsorbed gradient samples, on Fgn-pre-adsorbed gradient samples, on Fn-pre-adsorbed gradient samples, in 10%-serum-containing cell-culture medium, and on 10%-serum-pre-adsorbed gradients in cell-culture medium. The results are displayed in Figs. 5.4-5.7.

In order to probe the influence of the physicochemical properties of the mammalian-cell membranes themselves, in the absence of adhesion proteins, a control experiment was carried out by seeding hFFs in serum-free buffer on PLL-g-PEG gradient samples. The cell-adhesion density increased until a critical value \( n_{\text{EG}} = 6.2 \pm 0.5/\text{nm}^2, \frac{L}{2R_g} \approx 1 \), at which point a threshold was reached, while the spreading area showed a continuous increase along the gradient (shown in Fig. 5.4(a)).

Similarly to the study of serum-protein adsorption, the adhesion kinetics of hFFs were measured by seeding hFFs in 10% FBS-containing cell-culture medium on gradient samples for 0.5 hr, 1 hr and 4 hr. The results are shown in Fig. 5.5 (a). Unlike the serum-adsorption behavior, which displays a continuous increase as PEG surface density decreases (shown in chapter 4 Fig. 4.5), the adhesion density of hFFs at the investigated time periods increased from the highest EG density to lower densities, reaching a plateau that remained constant until 0 EG density was reached. The critical EG density to reach saturation seems to be time dependent, increasing from 5.3 ± 0.5/\text{nm}^2, to 7.8 ± 0.5/\text{nm}^2, and to 11.3 ± 0.4/\text{nm}^2 as the incubation time increases from 0.5 hr to 1 hr and to 4 hr, respectively. This finding might reflect the increased amount of adsorbed serum proteins along entire gradient samples as well as the capacity of hFFs to secrete ECM proteins for their own adhesion as incubation time increases. The trend of spreading area of hFFs along the gradients generally followed that of cell density after 0.5 hr, 1 hr and 4 hr incubation time, and the critical EG density to reach saturation was the same as that of cell-adhesion density.
Fig. 5.4  (a) hFF adhesion after 1hr-incubation in serum-free buffer along PLL-g-PEG gradient; (b) hFF adhesion after 1hr-incubation in DMEM along PLL-g-PEG gradient pre-exposed to 1 mg/mL BSA for 0.5hr.

To rule out the possibility of the influence of cell-secreted ECM proteins after seeding for 4 hr, a control experiment was carried out by firstly incubating PEG gradient samples with 10 % serum for 4 hr, followed by seeding hFF on this serum-pre-adsorbed gradient sample in serum-free DMEM for 1hr. The results are shown in Fig. 5.5(b). Compared with the results of 4 hr-incubation in Fig. 5.5(a), the hFF
adhesion density and the average spreading area were very similar. A difference lay in the absolute value of spreading area, which was higher than that after 4hr-incubation in Fig.5.5(a). This could be attributed to the different seeding-time period, as it usually takes a few hours (0.5-3h) for cells to spread and form a cytoskeletal filament assembly after becoming attached to the substrate.

![Graph showing cell adhesion and spreading area](image)

**Fig. 5.5** (a) hFF adhesion in cell-culture medium (10% FBS) for 0.5hr, 1hr and 4 hr; and (b) hFF adhesion after 1hr-incubation in DMEM along the PLL-g-PEG gradient pre-exposed to cell-culture medium (10% FBS) for 4hr.
Fig. 5.6  hFF (with or without RGD pretreatment) adhesion after 1hr-incubation in DMEM along PLL-g-PEG gradient pre-exposed to 50 ug/mL Fgn for 0.5hr. (a) cell adhesion vs. EG density; and (b) cell adhesion vs. Fgn surface density.

Corresponding to the measurement of BSA and Fgn adsorption, the initial adhesion behavior of hFFs was studied after pre-adsorption of BSA or Fgn onto PEG gradients in serum-free buffer for 1 hr. The results are shown in Fig. 5.4(b) and 5.6(a), respectively. For BSA-pre-adsorbed gradient samples, cell adhesion was highly inhibited (>90%) when the EG density was higher than 11.5 ± 1.0/nm². From this EG density, hFF density gradually increased until a low EG density 2.9 ± 0.6/nm², where
it saturated. The average cell-spreading area followed the same tendency with highly inhibited spreading at higher EG density, while as nEG decreased from 9.6 ± 0.7/nm² to 0, the spreading area increased continuously. Compared to the same conditions in 10 % serum, the spreading areas on BSA-pre-adsorbed samples were much lower, being only around half that at 0 EG density, while the cell density was similar. As for the cell response to Fgn-pre-adsorbed PLL-g-PEG gradients, the cell-adhesion density reached saturation even at high EG densities of 11.9 ± 0.7/nm². At this EG density the Fgn surface density was 25.2 ± 3.4 ng/cm². However, the saturation of the spreading area occurred at slightly lower EG density (10.7 ± 0.5/nm²), meaning a much higher Fgn surface density (58.4 ± 9.2 ng/cm²) to reach maximum spreading area. Below this EG density, hFF spreading area continuously increased as the EG density decreased.

Pre-incubation of hFF with cyclic RGD to block integrin-binding sites (e.g. αvβ3 integrins) led to a significant decrease in both adhesion density and spreading area of hFFs. The cell adhesion was greatly inhibited at high EG density and gradually increased as EG density decreased until nEG=5.1 ± 0.4/nm². The results of this adhesion-blocking assay suggest that an integrin-mediated process was involved in the hFF adhesion and spreading on Fgn-pre-adsorbed gradients. In addition, it appears that RGD-dependent integrins were more involved at high EG densities (above 6.7/nm²), compared to the behavior at low EG densities or on bare TiO₂.

On Fn-pre-incubated PLL-g-PEG gradients, the cell-adhesion behaviors (Fig. 5.7(a)) appear similar as on Fgn-adsorbed gradients. The cell density reached saturation at a little lower EG densities of 8.8 ± 0.6/nm², corresponding to a Fn surface density of 13.9 ± 3.2 ng/cm². Similar as for Fgn-adsorbed samples, the saturation of the cell spreading area for Fn-adsorbed samples occurred at slightly lower EG density (7.1 ± 0.5/nm²) compared to that for cell density, that is, a higher Fn surface density (20.6 ± 4.6 ng/cm²) to reach maximum spreading area compared to the critical Fn density for maximum cell density. Pre-incubation of hFF with cyclic RGD to block integrin-binding sites led to a significant reduction (ca. 30%) of both adhesion density and spreading area of hFFs for the entire range of EG density. Furthermore, at high EG densities (above 8.8 ± 0.6/nm²), both the values of the cell density and spreading area are higher compared to the results of control samples, which suggests other RGD-independent integrins were involved at higher EG density on Fn-adsorbed samples.

For both Fgn- and Fn-adsorbed gradient samples, the adhesion behavior was also studied with respect to the surface density of adsorbed proteins (shown in Fig. 5.6(b) and Fig. 5.7(b), respectively). Both the adhesion density and spreading area appeared to increase linearly with increasing adsorbed proteins until reaching a plateau.
Fig. 5.7  hFF (with or without RGD pretreatment) adhesion after 1hr-incubation in MEM along PLL-g-PEG gradient pre-exposed to 30 ug/mL Fn for 0.5hr. (a) cell adhesion vs. EG density; and (b) cell adhesion vs. Fn surface density.
5.2.3 Discussion

Compared to the trends for protein adsorption along PLL-g-PEG gradient surfaces, mammalian cell-adhesion density in serum-free buffer (Fig. 5.3(a)) shows a different adhesion profile—saturation occurring over the entire “mushroom” regime and significant inhibition in the “brush” regime. More interestingly, the average spreading area does not follow a similar trend to cell-adhesion density but rather a gradual decrease throughout the “mushroom” regime—similar to that seen for protein-adsorption behavior. Cell membranes, with their embedded proteins, are well positioned to attach to the substrate via multiple binding sites, each of which performs a specific interaction. It is likely that in the “mushroom” regime, as EG density increases, cells will tend to adapt their shape in order to achieve maximum adhesion density, reducing the contact area with the substrate and thus reducing the total steric repulsion force resulting from compression of PEG chains.

The hFF adhesion assay in serum-free buffer served as a control experiment to be compared with hFF adhesion on protein-pre-exposed PEG gradients. Both adhesion density and spreading area in 10% serum-containing medium increases significantly along the entire gradient surface, even at the onset of the “brush” regime. Compared to the kinetic study of serum adsorption, after 0.5hr, 1hr and 4hr, it is apparent that as the protein adsorption increases with incubation time, the n_{EG} value at which the saturation of hFF adhesion occurs also increases, reaching values as high as L/2R_{g}=0.73 after 4 h of incubation. This indicates the correlation of serum-protein adsorption and hFF adhesion, that is, serum-protein adsorption precedes hFF adhesion, especially in the high-n_{EG} regime.

hFF adhesion on BSA-pre-exposed gradients is similar to that in serum-free buffer, with a somewhat lower cell number and lower n_{EG} value at saturation, which may be due to the lower attraction force between BSA and hFF compared to the force between the TiO_{2} surface and hFFs. The adhesion behavior is different from that in 10 % serum-containing medium, with much lower nEG values at saturation and no saturation for the cell-spreading area. This suggests that BSA is unlikely to be responsible for the cell-adhesion behavior in 10 % serum-containing medium, consistent with the result of the relatively low affinity of BSA towards the TiO_{2} surface from the competitive protein adsorption study. Since BSA inhibits the blood clotting, which is beneficial for healing following implantation surgery, this may also be another benefit when using titanium as an implant material, in addition to its good mechanical properties and chemical inertness.
Unlike BSA, Fgn and Fn contain RGD sites that fibroblasts can attach to directly through integrin $\alpha_v\beta_3$ [96]. In order to quantify the effect of EG density and the amount of Fgn/Fn adsorption on both cell-adhesion density and spreading extent, a hFF adhesion assay was carried out on Fgn/Fn -preadsorbed PEG gradient surfaces. To determine to what extent specific ligand-receptor binding was involved, an adhesion-blocking assay was carried out by preincubation of hFF with cyclic RGD, so as to saturate the corresponding integrin-binding sites. For Fgn-adsorbed samples, adhesion-blocking results show that cell adhesion and spreading appears a maximal 50% decrease at $n_{\text{EG}}$ around 9.8/nm$^2$, whereas throughout the entire gradients reduction was much smaller. While on Fn-adsorbed gradient samples, the results of adhesion-blocking are different. Both the cell density and spreading area are decreased by ca. 30% throughout the entire range of PEG density, but the reduction at higher EG density (above 8.8/nm$^2$) is not as significant as that on Fgn-adsorbed samples. Therefore, the results suggest that a different adhesion mechanism may exist at higher EG density between Fgn- and Fn-adsorbed gradient samples, which may be related to the conformation change of proteins at different density of PEG. It was observed with AFM that Fn adsorption on hydrophilic and charged surfaces renders a significant conformational change compared to that in solution, from a compact, globular shape to an extended, rod-like shape [97]. The extension of the molecule is known to lead to the exposure of the RGD sequence, which thus becomes accessible for the relevant integrin receptors. On bare TiO$_2$, it is reasonable to assume that Fn also adopts a rod-like conformation, while as the EG density increases, the conformation changes and other binding motifs could be exposed on surface and become accessible for other RGD-independent integrin receptors (i.e. the V-region contains binding sites for $\alpha_4\beta_1$).

For Fgn-adsorbed samples, the saturation point occurred at high EG density (11.8/nm$^2$), in the “brush” regime, indicating that even a small amount of Fgn is sufficient for hFF adhesion and spreading. The saturation point for cell-adhesion density corresponds to a Fgn surface density of 25.2±3.4 ng/cm$^2$, and saturation of spreading area requires a rather higher Fgn surface density of 58.4±9.2 ng/cm$^2$. For Fn-adsorbed samples, on the other hand, the critical Fn surface density for the saturation of cell adhesion is significantly lower compared to that of Fgn. The critical Fn surface density for cell-adhesion density is 13.9 ± 3.2 ng/cm$^2$, and saturation of spreading area requires a Fn surface density of 20.6±4.6 ng/cm$^2$. It was reported that the spacing of ligands influences both cell spreading and cytoskeleton assembly [98-100]. In our system, after converting the surface-density value of Fgn, it turns out that an average spacing of 47±3 nm of Fgn is sufficient to allow integrin-mediated
fibroblast adhesion, and 31±3 nm for spreading, whereas for Fn, it is 72±3 nm for cell density and 59±3 nm for spreading. These results are roughly consistent with those acquired on other similar systems. For example, Danilov and Juliano adsorbed fibronectin and vitronectin onto tissue-culture-plastic surfaces and examined cell attachment. The results indicated a required RGD-to-RGD distance of <22 nm for Vitronectin and 42 nm for Fibronectin [101]. Similar studies with adsorbed cell-adhesion proteins have also been carried out by Underwood and Bennet, indicating a ligand-to-ligand spacing of 20 nm for Vitronectin and 37 nm for Fibronectin to achieve 75% BHK-21 cell adhesion [102]. Comparison of the results reported by Spatz and coworkers showing that an RGD lateral spacing of <58 nm is essential for cell-spreading and focal-adhesion dynamics [103], suggests that only a certain fraction of the adsorbed Fgn was available to the cells’ integrin-binding sites, possibly induced by denaturation of protein during interactions with the TiO₂ surface. The saturation of cell adhesion and spreading is believed to relate to the integrin-receptor availability and density on the cell membrane, and thus is cell-type dependent.

5.2.4 Conclusion

Overall, our results demonstrate a preceding and assisting effect of serum proteins and Fgn adsorption regarding cell adhesion and spreading behavior along PLL-g-PEG surface gradients on TiO₂ surfaces. This effect was more apparent in high-EG-density regions. BSA adsorption, in contrast, hardly affected cellular response compared to that of bare PLL-g-PEG/TiO₂ gradient surfaces. It can be concluded from our experiments that adhesion proteins are required for cells to adhere and spread in areas of high PEG density (even at the onset of the brush regime), since integrin binding plays an indispensible role in these regions. Finally, we also distinguished a critical value of fibrinogen and fibronectin spacing for integrin-mediated binding of fibroblasts.

5.3 Long-term Studies of Proliferation and Viability

5.3.1 Experimental

5.3.1.1 Cell Seeding and Nuclear Staining

A live stain of nuclei by Hoechst staining was applied one day before seeding cells onto gradient samples. After reaching confluence of 70-90%, cells in flasks were rinsed once with sterile PBS and then incubated with Hoechst stain 33342 (different
staining conditions were tested and optimized), and then rinsed twice with sterile PBS. After checking the nuclear staining by FM, the cells were maintained in the incubator over night. On the next day, cells were harvested by 0.25% Trypsin-EDTA treatment for less than 5 minutes in the incubator until they were detached, centrifuged for 5 minutes at 1000 rpm, re-suspended and diluted in serum-free medium (DMEM for hFF, and α-MEM for RCO). The seeding density was optimized to be 900 cells/cm² of hFF and 1500 cells/cm² of RCO for proliferation assays so that the adhered cells would not reach 100% confluence after 7-days incubation. Cells were allowed to adhere and spread for 1h in serum-free DMEM after seeding. Afterwards the medium was exchanged for growth medium (10% FBS supplemented DMEM for hFF, and 10% FBS supplemented α-MEM for RCO) for proliferation studies. Fluorescence images of the nuclei of adhering cells were taken at day 2, 4, and 7 to monitor adhering cell density. Each time after taking images, the samples were returned to the incubator until the next imaging date. The cell culture medium was changed every other day.

5.3.1.2 Phalloidin Staining

On day 7, the samples were removed from the incubator and gently rinsed with PBS (Fluka Chemicals, Buchs, Switzerland) solution pre-warmed to 37°C, and cells were fixed in 4% paraformaldehyde solution for 15 minutes and then gently rinsed twice with PBS. The adhering cells were permeabilized in 0.5% Triton X-100 in PBS (Fluka Chemicals, Buchs, Switzerland) for 5 minutes and then rinsed twice with PBS. Then were subsequently stained with Alexa Fluor 488 phalloidin (1:100 dilution in PBS; Invitrogen, Basel Switzerland) for 30 min. After the samples were rinsed twice with PBS, the gradient samples were mounted onto glass slides for fluorescence microscopy to obtain information of cell morphology.

5.3.1.3 Viability Staining

To check the viability and vitality of the adhering cells, at day 7 after seeding, a live/dead staining was carried out on other parallel sample groups by incubation with FDA/EtBr solution (diluted to 0.0025 mg/mL FDA and 0.005 mg/mL EtBr, both are from Invitrogen, Basel, Switzerland) for 3 minutes and then rinsed with PBS, after which fluorescence images were captured immediately, along the gradient samples.

All the above cell-adhesion assays were carried out in triplicate at each time and repeated at least once.
5.3.1.4 Imaging and Evaluation

All the fluorescence images were taken with an automated fluorescence microscope (Axio IMAGER M1m, Zeiss, Oberkochen, Germany). In order to monitor the proliferation of the adhering cells, three images were taken at 8 positions along the PLL-g-PEG gradient samples with a DAPI filter set (for Hoechst stain) at x10 magnification on day 2, 4, 7 after seeding. On day 7, images were taken with a Fluar 488 (for FDA) and a Fluar 546 (for EtBr) filter set at x20 magnification to investigate cell viability. Additionally, for other parallel sample groups on day 7, images were taken with a Fluar 488 (for Phalloidin) filter set at x20 magnification to obtain information of cell morphology. A total of at least 6 samples were investigated for each condition. For analysis, images of cell nuclei and cell morphology were processed with Image J software. All mean values are shown ± SEM (standard error of the mean). The level of significance p was set at <0.05.

5.3.2 Results and Discussion

5.3.2.1 Preparation experiments for nuclear staining of live cells

Unlike DAPI, Hoechst dye is cell-membrane-permeable, and thus it can be used to stain the nuclei of live cells to monitor cell growth on samples, if appropriate staining conditions are provided. Different concentrations of stain solution and staining time were tested to achieve the optimal conditions, such that the stained nuclei could show a sufficiently strong fluorescence signal and in the meantime, cellular activities were not influenced. Staining conditions are summarized in Table 5.2. hFF were seeded at 5000/cm² on day 0, on day 2 they were stained with different conditions and both the fluorescent microscopy images of nuclei and light microscopy images of cell morphology were captured on day 4 for analysis. Control samples are the cells without staining.

Table 5.2 Different staining conditions for Hoechst stain 33342. ☻ means more than 90% of the attached cells appeared normal spindle-shape morphology, rather than shrinking, round shape, whereas ☼ means the opposite. ✓ means the fluorescence intensity of nuclei is strong enough for eyepiece inspection and also camera capture, while × means the fluorescence signal was too weak.
<table>
<thead>
<tr>
<th>Conc. Time</th>
<th>1:1000</th>
<th>1:2000</th>
<th>1:4000</th>
<th>1:10000</th>
<th>1:20000</th>
</tr>
</thead>
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<tr>
<td>2 min</td>
<td>😊 ✓</td>
<td>😊 ✓</td>
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<td>1 min</td>
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<td>0.5 min</td>
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<td>😊 ⚹</td>
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<td>😊 ⚹</td>
</tr>
</tbody>
</table>

From the results summarized in Table 5.2, there are 4 staining conditions that meet the requirements, among which the attached cell density of the condition (1:10000, 1 min) was the highest. Therefore, this condition appeared to be the candidate for optimal staining conditions.

![Fig. 5.7](image-url)  
**Fig. 5.7** Examples of comparison of control samples with those treated with different staining conditions on day 4. (a) light microscopy image of control samples; (b) light microscopy image of samples of staining conc. 1:1000, and for 2 min; and (c) light microscopy image (left) and fluorescence image (right) of samples of staining conc. 1:10000, and for 1 min.

The optimal staining conditions (1:10000, 1 min) were checked by a WST-1 proliferation assay. Both stained cells and the control without staining were seeded at the same density 10000/cm² at day 0, absorbance of the solution in the well plates was measured at day 1 and day 2 at the wavelength of 450 nm. The results for comparison are shown in Fig. 5.8. There is little difference in cell proliferation between the stained and unstained cells. Therefore, the staining conditions appear to have almost no influence on cell proliferation and are thus appropriate for monitoring cell growth on gradient samples in live experiments.
Fig. 5.8 Absorbance of the solutions in the well plates with stained cells and unstained cells at the wavelength of 450 nm on days 0, 1, and 2.

5.3.2.2 Cell Proliferation and Viability on Gradient Samples

The adhered cell density was monitored over a 7-day period, and cell morphology and viability were measured at day 7, regarding the influence of different PEG density and conformation, as well as the effect of the pre-adsorbed proteins (Fgn, Fn, BSA and serum). Quantitative analysis of attached cell number at days 2, 4, and 7, and average spreading area of cells at day 7 are shown in Fig. 5.9 for hFF, and Fig.5.10 for RCO. The results are plotted against both EG density and surface density of pre-adsorbed proteins.

In Fig. 5.9, the adhered hFF density shows similar trends compared to that observed in the short-term adhesion experiment (Fig. 5.4-5.7), with similar EG density at the saturation point for each protein-backfilled gradient sample. However, cell density decreased dramatically in the transition position of high PEG density, compared to the behavior observed in short-term adhesion results, which means cells merely proliferate or cell growth was quite slow above a certain PEG density. For serum-backfilled gradient samples, it can be observed that cell density slightly increased at the position where the EG density is above 12.7/mm$^2$, whereas below this point, cell density starts to increase dramatically until reaching saturation. For Fgn- and Fn-backfilled gradients, the saturation occurred at the highest EG density, that is 17.6/mm$^2$ for Fgn and 16.4/mm$^2$ for Fn, while for BSA-backfilled samples, it occurred at lower EG density (9.4/mm$^2$). This difference could be due to the effect of the pre-adsorbed proteins. It was demonstrated in the last section that at higher EG density, adhesive protein (i.e. Fgn, Fn) adsorption is essential for cell adhesion, when integrin binding is involved, which leads to the formation of focal adhesions. It is known that focal adhesion could then influence cell signaling pathways, cell growth
and other functions. Therefore, for BSA-backfilled samples, a much lower EG density is able to inhibit cell growth greatly, compared to that of other-protein-backfilled samples.

The cell morphology for Fgn- and Fn-backfilled samples generally follows the trends of cell density. The saturation point occurred at EG density of 11.7/mm² for Fn, and 12.5/mm² for Fgn. However, for serum-and BSA-backfilled samples, the saturation point occurred at significantly higher EG density compared to the results of cell density, with 10.6/mm² for serum, and 9.4/mm² for BSA. This could be explained by the effect of PEG degradation during long-term cell culture, which leads to the loss of nonfouling properties, and thus proteins from the cell-culture medium and cell-secreted proteins could be deposited at high EG density, and enable cell spreading. Also, the results appeared that cell morphology were not significantly influenced by the pre-adsorbed proteins, but rather mainly by the EG density. The results of RCO (Fig. 5.10) were similar in these aspects.
Fig. 5.9 Adhered hFF density at day 2, 4 and 7, and cell spreading area at day 7, on (a) Fn-backfilled PLL-g-PEG gradients; (b) Fgn-backfilled PLL-g-PEG gradients; (c)
BSA-backfilled PLL-g-PEG gradients; and (d) serum-backfilled PLL-g-PEG gradients.
Fig. 5.10  Adhered RCO density at day 2, 4 and 7, and cell spreading area at day 7, on (a) Fn-backfilled PLL-g-PEG gradients; (b) Fgn-backfilled PLL-g-PEG gradients; (c) BSA-backfilled PLL-g-PEG gradients; and (d) serum-backfilled PLL-g-PEG gradients.
Fig. 5.11 An example of adhered RCO on gradient sample surfaces at day 7. The PLL-g-PEG gradients were backfilled with serum proteins prior to seeding cells. Images were taken at position 0mm, 6mm, 12mm and 18mm of a 20-mm long gradient sample. The nuclei were stained with the Hoechst dye, showing a blue fluorescence, while the cellular skeleton was stained with phalloidin, showing a green fluorescence.

Cell viability was characterized qualitatively with live/dead staining. For all the conditions under study, dead cells were rarely observed. Even at extremely high PEG density, more than 90% of adhered cells retain viability. An example is shown in Fig. 5.11. This result suggests that the PEG density under study does not lead to the apoptosis of cells in 7-day period, regardless of pre-adsorbed proteins.

Fig. 5.12 An example of adhered RCO on serum-backfilled gradient sample surfaces at day 7 after live/dead staining. Images were taken at position 0mm, 6mm, 12mm and 18mm of a 20-mm long gradient sample. Almost all the cells only show a green fluorescence, even at the very end of the samples with high PEG density, indicating a high ratio of live cells attached to the sample surfaces.

5.3.3 Conclusion

To conclude, we systematically studied the cell proliferation, morphology and viability in 7-day period, with respect to the influence of both PEG density and adsorbed proteins, with hFF and RCO. The adhered cell density after 7 days revealed a similar trend compared to that of 1hr-incubation, but with a more dramatic decrease at higher EG densities. Furthermore, cell morphology after 7 days did not quite correlate with the trend of cell density, which may be due to the degradation of PEG on the sample surface. Cell viability was hardly influenced by the existence of PEG, even at very high PEG density. Backfilling gradient samples with different proteins still resulted in different cell behavior, although not as significant as those in 1hr-incubation study. These results can be of direct benefit to innumerable implant-relevant biomedical applications—for example, by tuning the PEG surface density.
and adsorbing different proteins to modulate cell adhesion, spreading, proliferation and other functions.
Bacterial Adhesion on PLL-g-PEG Gradients

In this chapter, gradients were used for the study of microbial adhesion. This was a collaboration with Dr. Markus Schuppler’s group at the Institute of Food, Nutrition and Health, ETH Zurich. Bare PLL-g-PEG gradients and PLL-g-PEG gradients backfilled with different proteins were subjected to two strains of bacteria—*E. coli* and *S. aureus*—for short-term adhesion studies (1hr). The gradient fabrication, protein backfilling and sample characterization were carried out in our group, while bacterial assays were performed by our collaborators in their lab. The subsequent image capture and analysis were carried out by me using collaborators’ microscope. In this chapter, gradients were used for the study of microbial adhesion. This was a collaboration with Dr. Markus Schuppler’s group at the Institute of Food, Nutrition and Health, ETH Zurich. Bare PLL-g-PEG gradients and PLL-g-PEG gradients backfilled with different proteins were subjected to two strains of bacteria—*E. coli* and *S. aureus*—for short-term adhesion studies (1hr). The gradient fabrication, protein backfilling and sample characterization were carried out in our group, while bacterial assays were performed by our collaborators in their lab. I carried out image capture and analysis using their microscope.

6.1 Background of Bacterial Adhesion

Rather than leading a nomadic existence, bacteria prefer a surface-bound, community-based, sedentary lifestyle. The first observation of this tendency dates back to the beginning of the last century [104]. Their inclination towards surface adhesion appears to be a survival strategy for them to capture food in a nutritionally advantageous environment, with a biofilm to provide protection from external predators, dehydration, biocides, and other environmental extremes [105]. It is widespread in diverse ecosystems, and has been retained and refined over millions of years of evolution.
Bacterial attachment onto surfaces can be described as following several distinct phases [106]. Step (1), transportation of bacteria toward surfaces by long-range physical forces (i.e. gravitational and Brownian forces). Step (2), adhesion of bacteria onto surfaces, which is usually described in two distinct stages. “Reversible adhesion” occurs during the short period upon approaching the surfaces, and involves the physical-chemical forces between the cell wall and the surfaces. It can be then described by the extended DLVO theory, similarly to protein adsorption. “Irreversible adhesion”, which is stronger than in the reversible stage, involves covalent receptor/ligand-like binding. These interactions occur between the so-called adhesin molecules, mostly composed of proteins, located on bacterial cell-wall surfaces and appendages (i.e. flagella and fimbriae), and the corresponding biomolecules present on surfaces. Therefore, both the properties and features of bacterial cell wall and material surfaces play important roles in this stage. Many published works have addressed the influence of material surface properties in this aspect, such as surface topography, surface energy, hydrophobicity, charges and functionalities. Step (3), proliferation and synthesis of the biofilm matrix; and Step (4), biofilm maturation and detachment events. The life cycle of these attachment and colonization events is illustrated in Fig. 6.1 [106].

Fig. 6.1 Scheme of the biofilm development. Details of step 2 (“bacteria adhesion”).
Two separate stages are usually distinguished (“reversible adhesion” and “irreversible adhesion”) involving different physical-chemical and chemical bacteria/surface interactions [106].

Nowadays titanium and its alloys are widely used in dental and orthopedic implants. When the implants are introduced to the human body, different types of proteins and other biomolecules from body fluids adsorb onto the implant surfaces immediately. These subsequently influence not only the adhesion and growth of tissue cells, but also microbial adhesion and further biofilm formation. Thus an acute competition for adhering to limited surface areas takes place. It is known that the success of implant integration highly relies on the outcome of the race between tissue cells and bacteria for implant surfaces [107], as microbial adhesion dominating over tissue cell adhesion in the initial stage may later lead to infection, long-term inflammation, disintegration of implants and consequently the failure of implantation.

At a basic level, the adhesion mechanisms of eukaryotic cells and bacteria share many similarities, including the fact that they are both complex and multi-step processes occurring at the interface. For example, the intermolecular forces responsible for cell-surface binding, including non-specific extended DLVO forces and specific ligand/receptor interactions, are both important factors for initial attachment of both eukaryotic cells and bacteria. Furthermore, some of them could bind to the same types of biomolecules (ligands), e.g. *S. aureus* was reported to bind specifically to fibronectin, which is a ECM protein and serve as adhesive proteins for various types of tissue cells [108]. In this aspect, the bio-/physico-chemical properties of both material surfaces and cell/bacteria surfaces are involved in the outcome and thus need to be studied in depth. Understanding and differentiating between the adhesion behaviors of eukaryotic cells and bacteria is particularly important for advancing clinical implantation methodologies.

Developing biomaterials favoring tissue cell adhesion while inhibiting bacterial adhesion and colonization still appears to be a big challenge. In the past decade, a variety of strategies has been employed. For example, some hydrophilic polymer brushes (e.g. PEG) were shown to inhibit protein adsorption and bacterial adhesion, but unfortunately they also inhibit eukaryotic cell adhesion. Incorporating of ligand-mimetic peptides (i.e. RGD motif) selectively assists tissue cell adhesion to a certain extent. However, it is known that a single small peptide, in comparison to a whole ECM protein, is not able to achieve all the numerous functions ensuring the normal functioning of cells [109].
Previously, through the use of surface gradients of PLL-g-PEG on titanium oxide surfaces, a high-throughput, cost-effective screening tool, we carried out a quantitative study of serum-protein adsorption and subsequent tissue-cell adhesion, spreading, subsequent proliferation and viability. The results elucidated the underlying molecular mechanisms regarding tissue-cell response to biomedically relevant materials. We were also able to tune protein adsorption and fibroblast adhesion and spreading by varying PEG surface coverage. Through pre-adsorption of certain types of protein, initial cellular adhesion and subsequent proliferation were influenced up to certain PEG surface density.

In this chapter, by employing the same surface gradients, we investigated the cooperative effects of both PEG surface density and backfilling protein (albumin, fibrinogen, fibronectin, whole serum) in microbial adhesion, and the results were compared with those of tissue cells. The scope of this study is not only to understand the adhesion mechanisms of bacteria regarding the bio-/physico-chemical properties of materials surfaces at the molecular level, but also to reveal the possibility to modulate and thus to differentiate between cellular and bacterial adhesion behaviors by varying PEG density and backfilling with proteins, which is central in favorable race of adhesion between tissue cells and bacteria. *Staphylococcus aureus* was chosen for study as it is the most common infecting organism in orthopedic infection [110], and *Escherichia coli* was studied for comparison.

### 6.2 Experimental

#### 6.2.1 Sample preparation and characterization

Fabrication and characterization of PLL-g-PEG gradients is described in Chapter 3.3. The protocol for backfilling the generated PLL-g-PEG gradients with different types of proteins is described in Chapter 4.2. Three samples were fabricated at a time for each type of protein backfilling.

#### 6.2.2 Bacterial cultures

As representatives of Gram-positive and Gram-negative bacteria, strains of *Staphylococcus aureus* (ATCC 6538) and *Escherichia coli* (ATCC 35218) were precultured prior to performing the adhesion assays, according to the following protocol:
For *Staphylococcus aureus* a single colony was transferred from a BHI agar plate (Biolife; Milano, Italy) to a test tube containing 4 ml BHI broth (Biolife; Milano, Italy) and incubated at 180 rpm and 37°C overnight. A volume of 1 ml from the fresh overnight culture was added to 4 ml of fresh BHI broth and incubated at 180 rpm and 37°C for approx. 2 hours until an OD$_{600}$ of 1.8 (Libra 22 UV/Visible Spectrophotometer; Biochrom Ltd., UK), which corresponds to approx. $10^{10}$ CFU/ml. For *Escherichia coli* the procedure was similar to that for *Staphylococcus aureus* with the exception that Luria-Bertani (LB) broth (Merck Biochemicals, Germany) was used for culture. The bacteria were harvested in the late exponential growth phase by centrifugation for 10 min at 6000 g and resuspended in HEPES 2 buffer. This washing step was repeated once to eliminate residual substances and proteins. Finally, the washed bacteria were suspended in the designated buffer or solution.

### 6.2.3 Short-term adhesion assays

The bacterial concentrations were adjusted to be $10^9$/mL for the adhesion assays. The conditions were similar as those for eukaryotic cells short-term adhesion assays for the purpose of comparison. PLL-g-PEG gradients were placed in 5mL of bacterial suspension in different media and then kept at 37°C without shaking for the designated period of time (1hr). Different assay conditions are summarized in Table 6.1.

All of the bacterial-adhesion assays were carried out in triplicate at each time and repeated at least once.

**Table 6.1** Conditions used for bacterial-adhesion assays, for both *E.coli* and *S. aureus*.

<table>
<thead>
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<th>No. of assay</th>
<th>gradient pre-exposure</th>
<th>Adhesion Medium</th>
</tr>
</thead>
<tbody>
<tr>
<td>a</td>
<td>no</td>
<td>HEPES 2 Buffer</td>
</tr>
<tr>
<td>b</td>
<td>50 µg/mL Fgn</td>
<td>HEPES 2 Buffer</td>
</tr>
<tr>
<td>c</td>
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</tr>
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<td>d</td>
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</tr>
<tr>
<td>e</td>
<td>10% serum</td>
<td>HEPES 2 Buffer</td>
</tr>
<tr>
<td>f</td>
<td>no</td>
<td>HEPES 2 Buffer containing 10% serum</td>
</tr>
</tbody>
</table>
6.2.4 Staining

After incubation for 1hr, the bacteria suspensions were exchanged with PBS solution gently and this rinsing step was repeated once to remove non-adherent bacteria. Then the adherent bacteria on the samples were fixed by immersion in 4% PFA for 15min. After rinsing with PBS and subsequently with DI water, samples were air-dried and then an ethanol extraction with rising concentrations (15%, 80%, 100%) was performed. Adherent bacteria were then stained by covering the sample with 40 µl DAPI for 15 min and then rinsing with PBS. This fixation and staining protocol is a modified method of fluorescence in situ hybridization (FISH) of bacteria.

6.2.5 Imaging and evaluation

The fluorescence images of bacteria were taken with an epi-fluorescence microscope (Zeiss Axioplan, Carl Zeiss AG, Germany). In order to investigate the density of the adhering bacteria, three images were taken at 8 positions (3 mm apart, the same as in Chapter 3.3) along the PLL-g-PEG gradient samples with a DAPI filter set, and at x20, and x40 magnification. A total of at least 6 samples were measured for each condition. For analysis, images of fluorescently stained bacteria were processed with ImageJ software for automatic counting of bacteria number. Comparisons between measurement series were made using nonparametric tests because variances were not homogeneous and observed values were not distributed normally, even after appropriate transformations. All mean values are shown ± SEM (standard error of the mean). The level of significance p was set at <0.05.

6.3 Results and Discussion

The results of adhered density of bacteria following 1h-incubation without shaking are shown in Fig. 6.2 for S. aureus, and Fig. 6.3 for E.coli. For protein-backfilled PLL-g-PEG gradient samples, the results were plotted against the monomer EG density as well as the surface density of adsorbed proteins. For other samples without backfilling of proteins, the results were plotted against only the surface density of EG monomer.

Compared with the trends of tissue-cell adhesion along PLL-g-PEG gradient surfaces (Fig. 5.4-Fig. 5.7), bacterial adhesion density in buffer (Fig. 6.2 and Fig. 6.3) shows a different adhesion profile—no saturation occurring throughout the entire
PEG density for all the assay conditions under study, but rather a gradual decrease as EG density increases, which is similar to that seen for protein-adsorption behavior. The difference is more significant for the condition of bare PLL-g-PEG gradients without backfilling of proteins (Fig. 6.2(a) and Fig. 6.3(b)). This difference between tissue cells and bacteria may be related to the difference of rigidity between the eukaryotic cell membrane and the bacterial cell wall. It is known that, due to the difference of composition, bacteria are much less deformable than eukaryotic cells and would usually maintain their shape when attaching to a surface [106]. Therefore, for eukaryotic cells, it is likely that in the “mushroom” regime, as EG density increases, cells will tend to adapt their shape in order to achieve maximum adhesion density, reducing the contact area with the substrate and thus reducing the total steric repulsion force resulting from compression of PEG chains. While for bacteria, they can hardly change the original shape, limiting the possibility to sense and adapt to the inert patches on sample surfaces. Thus, even a quite low EG density in the “mushroom” regime has a significant inhibition effect on microbial adhesion. More interestingly, adhesion of *S. aureus* decreases more dramatically as EG density increases, and requires lower EG density (6.9/mm²) for inhibition of adhesion to large extent (>90%), compared to that for *E. coli* (9.2/mm²). It is either due to the difference of rigidity as the cell wall of *S. aureus* should be more rigid, or the fimbriae involved, which are adhesion organelles expressed by many Gram-negative bacteria, or both.

The bacterial adhesion assay on bare PLL-g-PEG gradients without backfilling of proteins in buffer serves as a control experiment to be compared with that on protein-pre-exposed PEG gradients (Fig. 6.2 (b)-(e) and Fig. 6.3(b)-(e)). It can be observed that, for all the backfilling proteins under study, the adhered density of both *S. aureus* and *E. coli* dramatically decreases throughout the entire PEG density, and especially in the “mushroom” regime. Among all of the 4 types of proteins under study, this effect is especially significant with serum-protein backfilling, which inhibits *S. aureus* adhesion by ca. 99%, and *E. coli* by ca. 90%, even at 0 EG density. The inhibitory effect of the other backfilling proteins on adhesion appeared to be bacteria-type dependent. For *S. aureus*, the adhesion behaviors are quite similar for Fn- and BSA-backfilling (maximum inhibition by ca. 95%), while Fgn-backfilling has less effect on adhesion inhibition (maximum inhibition by ca. 82%). For *E. coli*, the influence of the other three proteins appears to differ only slightly (by ca. 50%), and the sequence regarding the inhibitory effect is Fgn>Fn>BSA. It was reported that pre-coating of some surfaces with certain proteins (i.e. BSA, serum) significantly inhibited bacterial adhesion [111-113], and it was proposed that the inhibitory effect of serum is mainly due to the presence of apo-transferrin [113]. Our results are in agreement with this.
published work. Furthermore, we also distinguished to which extent the type and the amount of pre-adsorbed proteins and the PEG density could inhibit the initial microbial adhesion.

Interestingly, the effect of the pre-adsorbed proteins on eukaryotic cell adhesion completely contrasts with their effect on microbial adhesion. For eukaryotic cells, while backfilling with BSA merely influences the adhesion density and spreading area, pre-adsorbed Fgn, Fn and serum proteins all promoted cell adhesion density. Spreading area and cell adhesion could reach saturation in the low-$n_{\text{EG}}$ regime and could at least be improved to a certain extent in the high-$n_{\text{EG}}$ regime. Also, previous long-term studies indicates that cell viability is hardly influenced by the existence of PEG, and only very high $n_{\text{EG}}$ values have a negative effect on cell proliferation. These different adhesion behaviors on protein-adsorbed PLL-g-PEG gradients between eukaryotic cells and bacteria could provide a means to develop strategies to improve the behavior of implants, concerning the race between tissue cell and bacterial adhesion.

Another control experiment was carried out by incubation of bare PLL-g-PEG in buffer containing 10% serum, so as to be compared to the condition of serum-backfilled samples in buffer. The results for *S. aureus* and *E.coli* are shown in Fig. 6.2(f) and 6.3(f), respectively. Upon comparison, it can be observed that although the bacterial adhesion still decreases a lot compared to the condition (a) of bare PLL-g-PEG in buffer, the effect is not as much as pre-adsorbed serum proteins in condition (e) in the “mushroom” regime. Incubation of protein and bacteria together in the buffer solution at the same time only inhibits *S. aureus* adhesion by ca. 83% even at 0 EG density, and by ca. 33% for *E.coli*. The decrease of the inhibition effect indicates that there exists a competition between serum protein adsorption and bacterial adhesion in occupying the free positions on sample surfaces, regarding the kinetic aspects. A study of such competition between protein and bacterial adsorption would be useful and of direct benefit regarding in vivo behavior of biomaterials.
Fig. 6.2  S. aureus adhesion after 1hr-incubation along gradient samples under different assay conditions corresponding to section 6.2.3. (a) S. aureus adhesion on PLL-g-PEG gradient samples in HEPES 2 buffer; (b) S. aureus adhesion on Fgn-pre-incubated PLL-g-PEG gradient samples in HEPES 2 buffer; (c) S. aureus adhesion on Fn-pre-incubated PLL-g-PEG gradient samples in HEPES 2 buffer; (d) S. aureus adhesion on BSA-pre-incubated PLL-g-PEG gradient samples in HEPES 2 buffer; (e) S. aureus adhesion on serum-pre-incubated PLL-g-PEG gradient samples in HEPES 2 buffer; and (f) S. aureus adhesion on PLL-g-PEG gradient samples in HEPES 2 buffer containing 10% serum.
Fig. 6.3 *E. coli* adhesion after 1hr-incubation along gradient samples under different assay conditions corresponding to section 6.2.3. (a) *E. coli* adhesion on PLL-g-PEG gradient samples in HEPES 2 buffer; (b) *E. coli* adhesion on Fgn-pre-incubated PLL-g-PEG gradient samples in HEPES 2 buffer; (c) *E. coli* adhesion on Fn-pre-incubated PLL-g-PEG gradient samples in HEPES 2 buffer; (d) *E. coli* adhesion on BSA-pre-incubated PLL-g-PEG gradient samples in HEPES 2 buffer; (e) *E. coli* adhesion on serum-pre-incubated PLL-g-PEG gradient samples in HEPES 2 buffer; and (f) *E. coli* adhesion on PLL-g-PEG gradient samples in HEPES 2 buffer containing 10% serum.
6.4 Conclusion

In summary, we carried out short-term bacterial adhesion studies on protein-backfilled PLL-g-PEG gradient samples. We were able to distinguish the effect of the type and the amount of pre-adsorbed proteins, in conjunction with varied PEG density, on the initial microbial adhesion. The adhesion behavior of bacteria on gradients with/without protein backfilling was significantly different from that of eukaryotic cells described in the last chapter. This difference may be related to the different physical properties of the eukaryotic cell membrane and the bacterial cell wall, as well as different adhesion mechanisms. The use of PLL-g-PEG gradient samples helped to differentiate the adhesion behaviors between eukaryotic cell and bacteria, which could be a promising avenue for improving the behavior of implant biomaterials, and influencing the race between tissue cell and bacterial adhesion.
This chapter contains the conclusions and outlook of Project 1, summarizing the work and results in chapter 3-6, and providing ideas for future work related to this project.

7.1 Conclusions

The surface-chemical gradient fabrication technique by a controlled dipping process presented in chapter 3 has been demonstrated to be a well-established, facile method allowing for the reproducible production of many gradient samples at a time and in one step, using VASE and FM as non-destructive and straightforward techniques for sample characterization. The aim of this project was thus to employ the generated gradients—high-throughput, cost-effective tools—for biological applications.

PEG brushes are shown to be non-biofouling surface coatings. Tuning the density of surface-exposed PEG chains may influence protein adsorption and cell adhesion. Through the use of PLL-g-PEG-density gradients on titanium oxide surfaces, we were able to carry out a systematic and quantitative study of bioadhesion (protein, tissue cell, bacteria), to further understand adhesion mechanisms at a molecular level and subsequent modulate adhesion behaviors for implant-related biomedical applications.

The adsorption behavior of single proteins (Fgn, Fn, Ab) was investigated with VASE and FM. The results generally correlated with proposed semiempirical geometrical models, illustrating the effect of the PEG-chain surface density and conformation on the inhibition of protein adsorption. Distinct differences could be observed between individual adsorbing proteins and may be attributed to their mode of surface attachment. Competitive adsorption indicated Fgn adsorption dominated over Ab adsorption along the entire PLL-g-PEG gradient samples in minutes to hours.

Short-term cell-adhesion assays were carried out with hFF to further elucidate the underlying mechanism of cell adhesion and spreading as a function of PEG coverage
and amount of the pre-adsorbed proteins. The influence of these factors on cell adhesion could be directly observed, and insights gained into the roles of both nonspecific binding and specific integrin binding in cell adhesion. Cell proliferation and viability were studied with hFF and RCO, indicated the limited inhibitory effect of PEG density compared to that on short-term adhesion, possibly due to the biodegradability of PEG. Different pre-adsorbed proteins showed slightly different extents of effect.

Microbial adhesion was also investigated on these types of gradient surfaces, with respect to the combinatory effect of PEG density and protein-backfilling. Differently from tissue cell adhesion, it showed that protein backfilling inhibited bacterial adhesion throughout the entire PEG densities, especially in the “mushroom” regime. Among the 4 types of pre-adsorbed proteins under study, serum showed the most significant inhibitory effect for both *S. aureus* and *E. coli*. This contrasting behavior may suggest a promising strategy for tuning PEG density and pre-adsorbed proteins to differentiate tissue cell and bacterial adhesion behaviors and influence the race between these two classes of species.

**7.2 Outlook**

The differences observed between individual adsorbing proteins along gradient samples may have resulted from their mode of surface attachment, especially the orientation/conformation change upon adsorption, considering the influence of physico-chemical properties of the substrate (TiO₂) and the PEG density. The study of this could be essential for further elucidating and confirming the factors influencing protein adsorption, in particular for Fgn and Fn. Several published works have employed the AFM technique for direct imaging of Fgn and Fn on different substrates (i.e. mica and silicon wafer). Although the imaging process might be time-consuming, and sometimes only works for low coverages of proteins, it could still be worth trying in the present case, especially with the use of the ultraflat Ti surface described in Chapter 8. The results could not only correlate with the amount of adsorbed proteins reported in chapter 4, but also the tissue-cell adhesion behaviors in chapter 5, and even bacterial adhesion behaviors in chapter 6.

Regarding competitive protein adsorption, it would be important to study individual proteins (i.e. Fgn, Fn) labeled with fluorescencs, versus serum and plasma proteins, especially for *in vivo* applications of biomaterials, where Vroman effects could play an important role. Some preliminary experiments showed that 50 µg/mL Fgn and 30
µg/mL Fn could not compete with 5-10% serum along the entire PEG gradients within 10 mins to 1hr as detected by FM. Dilution of serum to 2% started to reveal a certain extent of competition between Fgn (or Fn and serum proteins) on bare TiO₂. Also, the measurement can be optimized by using in situ monitoring techniques, such as TIRFM, as some published work showed a quick replacement within 5-10 minutes. Thus, PEG gradients can also be used for a systematic investigation of competitive adsorption of individual proteins versus serum/plasma depleted of this protein.

Our results with tissue-cell adhesion were a synergic effect of PEG density and pre-adsorbed protein. However, the amount, and possibly the conformation/orientation of the protein were also dependent on PEG density. Thus, it is hard to separate the effects of these two when using gradient samples. Using several individual samples of different PEG density but of the same protein density may be a good solution. And the value of protein density under study can be varied from below to above the saturation level.

We have studied cell proliferation and viability over a 7-day period. However, cell differentiation is also an important factor regarding implant-related applications. We performed some preliminary experiments to test the possibility of using immunofluorescence staining with specific antibodies to quantify the differentiation of osteoblasts. ALP activity is an early marker of differentiation. Our tests at EMPA showed very similar results with very weak signals on both control (treated with only proliferation medium) and experimental samples (treated with differentiation medium) on bare TiO₂ substrates at days 4, 7 and 10, indicating the antibody is not very specific to the ALP of RCOs, and thus more effort needs to be made to try other antibodies. Osteocalcin secretion is a late marker of osteoblastic differentiation. Although the antibody and staining [114] worked for the RCOs at day 7 and 10 on a bare TiO₂ substrate, a question mark needs to be put over the quantitative results on gradient samples. As the cell spreading also changes along gradients, RCO shrinking in the “brush” regime, the intensity of fluorescence may not be an accurate indication of the total amount of osteocalcin. Ex-situ measurement and analysis using cell lysate might be necessary for quantification of differentiation.

Bacterial adhesion is a first step, but subsequent biofilm formation is also important to study using PEG gradients with pre-adsorbed proteins, using VASE and SEM characterization. Furthermore, regarding the race between tissue cell and bacterial adhesion, it would be necessary to carry out a competitive adhesion assay with different ratios of tissue cell vs. bacteria at the same time in solution on these gradients. The results could then be of direct relevance for clinical application.
Chapter 8

Development of Patterned Ultraflat Surfaces

In this chapter, a reproducible preparation method of patterned, ultraflat surfaces of composite metals is described. A modified template-stripping method in combination with different surface-patterning techniques was investigated for optimization of preparation conditions. Examples of Au/Ag and Ti/Au patterns were demonstrated and compared. The stripped surfaces were characterized by means of several surface-analytical techniques—AFM imaging for surface morphology, XPS and ToF-SIMS for surface chemistry.

8.1 Ultraflat Surfaces

Surfaces with roughness of less than 1-nm root mean square (rms) are known as ultraflat surfaces, among which mica and silicon wafer surfaces are the most common materials. They have been intensively employed in research, especially in scanning probe experiments, e.g. studying silane-monolayer formation, as well as DNA and protein adsorption [115-117]. However, as for most of other materials of interests, such as gold and titanium, which are frequently used not only in fundamental research but also in industrial applications, evaporation or sputtering does not generally achieve exceptional flatness, but often generates small irregular grains with several-nanometer roughness, even with slow deposition rates and further annealing processes.

Several strategies have been developed to fabricate ultraflat surfaces of different materials, among which, template stripping is a simple yet effective method. Through the use of existent ultraflat surfaces as templates, such as mica and silicon wafers, atomically flat surfaces of other materials can be generated by either mechanical stripping, or chemical stripping [118]. The success of this method relies on the low adhesion force between the template and the desired material. Ultraflat Au, first reported by Hegner et al. [118], is the most frequently generated surface with the
template-stripping method. It has been previously used to reveal the island microstructure of alkanethiol gradients by means of AFM imaging [119].

There are several limitations and challenges in the current template stripping method: (1) For the mica template, there is a strong possibility that residues from the templates remain on the surface after stripping, as the sheet-like structure breaks easily [120]; (2) For certain materials (i.e. Ti), the adhesion force at interface is too high for the two surfaces to be readily separated in the stripping step, or, even if separation occurs, incomplete stripping can generate surfaces with significant defects; (3) The preparation of ultraflat surfaces of composite materials of particularly challenging, as more than one material is present in the surface. It thus requires one more condition that the stripping should be compatible with the different adhesion forces between the different materials and the template.

As the adhesion between titanium and silicon is very strong, to our knowledge there has been no credible report yet on complete stripping to generate ultraflat Ti surfaces, but with silicon residues detected using common template stripping process reported by Rosstti et al. for the XPS measurement on the stripped Ti samples prepared by Cacciafesta et al. [120,121], not to mention the preparation of ultraflat surfaces of composite materials with Ti. Here, we developed a modified template-stripping method to generate Ti/Au patterned ultraflat surfaces, with an additional step of silanization on the template (silicon wafer) to reduce the adhesion force while not increasing the roughness. The generated sample surface can be as large as several square centimeters, free of residues from the silicon templates, robust enough to tolerate common solvents such as water and ethanol, and this method can work with different area ratios of Ti vs. Au (even 100% Ti surfaces).

8.2 Preparation Method

The preparation method involves a silanization step on the template (silicon wafer) to reduce the adhesion force, a surface-patterning technique to generate the contrast of the different materials, and a mechanical-stripping process to separate the desired ultraflat surfaces from the silicon-wafer template. A schematic drawing of the general preparation procedure is illustrated in Fig. 8.1. Conditions of silanization, lithography methods, and lift-off protocols were tested and optimized. In some situations (perfluorinated silane for photolithography, and two different silanes for particle
lithography), the sequence of the silanization and lithography steps had to be exchanged.

Fig. 8.1  Schematic of the preparation method for ultraflat surfaces with patterned Ti/Au.

8.3 Experimental

8.3.1 Materials

4” Silicon wafers (100) were purchased from Si-materials (Landsberg/Lech, Germany). Hexamethyldisilizane (HMDS, ≥99%) and 1H,1H,2H,2H-Perfluorooctyltrichlorosilane (PFOCTS, 97%) were obtained from Sigma-Aldrich Chemicals (Karlsruhe, Germany). Gold and Titanium (both 99.99%) were purchased from Umicore Materials AG (Balzers, Liechtenstein). Microposit S1818 photoresist and MF-319 developer were obtained from Rohm and Haas Electronic Materials Schweiz GmbH (Luzern, Switzerland), and LOR 5B was purchased from Micro Resist Technology GmbH (Berlin, Germany). UV curing optical adhesive NOA 61(or 63) was purchased from APM Technica AG (Heerbrugg, Switzerland). Monodispersed polystyrene particles (5 µm and 0.5 µm, respectively) were obtained from Microparticles GmbH (Berlin, Germany), and monodispersed silica microspheres (2.5 µm) were purchased from Polysciences, Inc. (Warrington, England). All solvents were HPLC grade and obtained from Sigma-Aldrich (Buchs, Switzerland) unless otherwise mentioned.
8.3.2 Preparation Procedure

8.3.2.1 Cleaning and Silanization of Silicon Wafer Surfaces

Photolithography: A 4” silicon wafer was first cleaned by immersion in freshly prepared Piranha solution (7:3 v/v, 97% H$_2$SO$_4$:30% H$_2$O$_2$) for 30 min (Caution: Piranha is an extremely dangerous oxidizing agent and all protective measures must be adopted to prevent any harm!) and followed by a thorough rinse with MilliQ water and blow drying in N$_2$. Afterwards the wafer was activated by oxygen plasma (Harrick, Ossining, NY, USA) for 1 min and then immediately subjected to vapor-phase silanization with silane solution in a desiccator connected to a roughing pump, to form a passivation layer. Different silanes were tested and silanization conditions were optimized.

Particle lithography: Silicon wafers were diced into pieces of 1.5 cm x1.5 cm for tilt-drying protocol, and 3 cm x 3 cm size for spin-coating protocol by a sawing machine (POWATEC GMBH, Hünenberg, Switzerland). Prior to use, the substrates were cleaned by the following protocol: (i) 2x10 min sonication in toluene, (ii) 2x10 min sonication in 2-propanol and (iii) 2 min oxygen-plasma cleaning in a plasma cleaner/sterilizer PDC–32G instrument (Harrick, Ossining, NY, USA) immediately before particle self-assembly process.

8.3.2.2 Lithography for surface patterning

Photolithography for mm-µm patterning: two protocols were tested, including

(a) 1-layer photoresist: A standard photolithography protocol was used. A silanized silicon wafer was spin-coated with a ca. 1 µm-thick layer of S1818 positive photoresist (@1000 rpm for 3 s for spreading and then @3000 rpm for 60 s), followed by soft-bake @ 100°C for 2 min on a hotplate. After exposure to UV with constant intensity (100 mJ/cm$^2$ @405 nm) through a chromium/quartz mask in vacuum-contact mode with a mask aligner (Karl Süss MA6, Süss MicroTec AG, Germany), samples were then developed in MF-319 for 1-1.5 min with gentle agitation, followed by a thorough rinsing with DI water and then blow drying in N$_2$.

(b) Bi-layer resist: A bare silicon wafer was spin-coated with ca. 200 nm-thick LOR 5B resist layer (@ 500 rpm for 3 s for spreading and then @3000 rpm for 60 s), baked
@ 170°C for 5 min on hotplate, and then spin-coated with a layer of S1818 photoresist. The rest of the procedure was the same as in (a). After developing for ca. 75 s, samples were rinsed with DI water and then blown dry in N₂. Afterwards they were ashed by oxygen plasma (Harrick, Ossining, NY, USA) for 2 min, and then immediately subjected to silanization if necessary (in the case of perfluorinated silane) prior to metal deposition.

**Particle lithography for sub-μm to nm patterning:** Particle solutions were sonicated for 10 min for homogeneous dispersion each time prior to use. Depending on the size of the particles, different approaches were used, including

(a) for μm-size: a tilt-drying protocol was used, and the concentration of particles, as well as the tilt angles were optimized to achieve monolayer coverage of large area. A 1.5 cm x 1.5 cm silicon substrate was placed at a tilt angle of 5°. Around 45 μL of 2%wt polystyrene (PS) particle (5 μm) solution in water, or 1%wt silica spheres (2.5 μm) were placed onto the entire silicon substrate and then allowed to dry and self-organize into ordered arrays as the solvent water gradually evaporated.

(b) for sub-μm size: a spin-coating protocol was used, and the concentration of particles, as well as the spin speed, were optimized to achieve monolayer coverage over a large area. A 3 cm x 3 cm silicon substrate was placed on a spin coater. Around 250 μL of 10%wt PS particles (0.5 μm) suspension in water was placed on the substrate and then immediately ramped up in speed for 1 s and spin-coated @ 1000 rpm for 3 min.

After the samples were completely dry, they were activated by oxygen plasma for 20 s, and then immediately subjected to vapor-phase silanization.

**8.3.2.3 Metal deposition**

The patterned samples were mounted and introduced into the E-beam metal-evaporation system (Plassys MEB550SL, PLASSYS-BESTEK, France). At a pressure below 10⁻⁷ mbar, 50 nm of titanium was first deposited at a rate of 0.1nm/s and followed by deposition of 50 nm of Au for prevention of Ti from oxidation. The above processes of photolithography and metal deposition were carried out under clean-room conditions.
8.3.2.4 Lift-off process

Wafers were cut into pieces of designated size with a diamond knife, and then subjected to the lift-off process.

Photolithography: For 1-layer resist, samples were immersed in acetone for around 2 hr, followed by gentle sonication (50% intensity for 3 min, Sonorex Super 10 P, BANDELIN electronic, Berlin, Germany) if necessary until the metal film was removed and patterns could be clearly observed. In the case of bi-layer resists, the samples were immersed in NMP (1-methyl-2-pyrrolidinone) @50°C until the metal film was detached from the silicon wafer and patterns could be clearly observed, and then a final cleaning step was carried out in fresh NMP with gentle sonication (30% intensity for 1 min), followed by immersion in isopropanol (IPA) with gentle agitation for 5 min. The process was repeated once with fresh IPA to remove NMP, and finally a thorough rinse with IPA and blow drying in N₂.

Particle lithography: Different lift-off protocols were tested, including

(a) Sonication in different solvents (toluene, 30% isopropanol/water, water) for various times until most of the colloids were removed, as observed by means of light microscopy.

(b) Scotch-tape stripping (several times at different directions) followed by sonication in organic solvent (2x 3min in acetone and then 2 min in isopropanol) to remove residues of tape, and afterwards a final ashing step with oxygen plasma for 2 min.

(c) (a) together with further cleaning step of plasma or UV/ozone or (b) together with further cleaning step of plasma or UV/ozone.

8.3.2.5 Gold deposition for backfilling

Samples were backfilled with 150 nm of Au in a metal-evaporation system (Bal-Tec MED020, Bal-Tec AG, Balzers, Liechtenstein) at a pressure below 2*10⁻⁵ mbar. The sample stage was tilted at an angle of 30° and a spin speed of ca. 200 rpm was used during gold deposition. For the first 10 nm of gold film, the deposition rate was controlled to be below 0.05 nm/s.
8.3.2.6 Gluing and stripping

The gluing process was performed according to the instructions provided by the manufacturer. An appropriate amount of UV glue NOA 61 (or 63) was applied onto the samples, and cleaned glass slides (cut into pieces of designated size and then sonicated in isopropanol for 20 min prior to use) were carefully attached to the sample surface, and the glue cured under UV light for 20 min. The samples were left for aging in an oven overnight at 50°C to achieve optimum adhesion, followed by cooling down for at least 12 hr prior to the mechanical stripping process. The template-stripping step was performed by carefully scratching the edges of the silicon wafer glued to the glass slide with a cutter and the metal surface was then separated from the silicon substrate by pressing the substrate and pulling up the glass slide with two tweezers at the same time.

8.3.3 Characterization

CA

The static contact-angle measurements of the silicon substrates subjected to silanization were carried out with a NRL contact-angle goniometer (model 100-00-230, Ramé Hart Inc., NJ, USA) by placing a 4 µL water droplet onto the substrates.

VASE

The thickness of the generated silane adlayer on silicon surfaces was characterized by VASE (M-2000F, L.O.T. Oriel GmbH, Germany), equipped with a focusing probe. Measurements were conducted under ambient conditions at three angles of incidence (65°, 70°, and 75°) in the spectral range of 370–1000 nm. Spectroscopic scans were taken before and after silanization. Measurements were fitted with the WVASE32 analysis software using a multilayer model for a silicon dioxide layer of 2.3 nm on silicon (1 mm) and a silane adlayer, of which the thickness was determined by a Cauchy model ($An=1.45$, $Bn=0.01$, and $Cn=0$).

Light Microscopy

After being subjected to each step of lithography, lift-off, and stripping, the samples were first checked by light microscopy (Axio IMAGER M1m, Zeiss, Oberkochen, Germany) at different magnifications (x4, x10, x20, x60).
AFM

AFM topographical imaging was carried out on a DI AFM Nanoscope Dimension 3100 (Veeco, USA) with Nanoscope version 7.2 software. Tapping mode was performed in air using silicon cantilevers with a resonance frequency of ca. 300 kHz (Veeco, USA), at a scan speed of 10-20 µm/s. Images of large areas (>5 µm) were subjected to first-order flattening, but small-area images were not processed.

Adhesion-force mapping was carried out using an AFM Asylum MFP 3D (Asylum Research, Santa Barbara, USA). By choosing a 10 µm*10 µm area on the sample surface, 100 force-distance curves were recorded. The recorded curves were then plotted as a histogram to calculate the mean and standard deviation of the adhesion force. The Si₃N₄ cantilevers used for the measurement were purchased from Veeco (USA) and had a spring constant of ca. 0.6 N/m. Prior to measurements, the cantilevers were subjected to UV/ozone cleaning for 20 minutes.

ToF-SIMS

ToF-SIMS measurements were carried out with a ToF.SIMS² spectrometer (ION-TOF GmbH, Munster, Germany) in the mass range 0–1000 m/z. Both spectral and imaging acquisition were carried out with Bi+ primary ions, at an energy of 25 kV and a current of ca. 0.75 pA for spectra and ca. 0.25 pA for imaging. All spectra were acquired with high mass resolution (≥5000) and characteristic peaks of negatively charged secondary ions were identified. The imaging was performed in the so-called “burst alignment” mode, providing good lateral resolution typically around 300 nm and unit mass resolution in a reasonable acquisition time. All surfaces were scanned with an ion-dose density of 5*10¹²/cm² across a 100*100 µm² area for Au/Ag and Ag/Au samples, and 200*200 µm² area for Ti/Au samples. For each sample, three positions were randomly chosen. At least two different batches were measured and 2 samples were examined from each batch. All data were processed and analyzed with IonSpec and IonImage software (ION-TOF, version 4.1). Negative spectra were calibrated using the C−, CH−, C₂H− and Au₃− peaks, and positive spectra using the C+, CH+, CH₃+, C₂H+, and C₃H₆+ peaks.

XPS
XPS survey spectra on template-stripped Ti samples were acquired with a XPS Sigma 2 instrument (ThermoFisher Scientific, Loughborough, Great Britain). It is equipped with a UHV chamber (pressure<10^-6 Pa during measurements), a non-monochromated 300 W Al Kα source (hν=1486.6 eV) that illuminates the sample at an angle of 54° to the surface normal, a hemispherical analyzer mounted at 0° with respect to the surface normal, eliminating the need for angular distribution correction, and a detector consisting of 7 channeltrons. The spot size of the analyzed area (large-area mode) was 400 µm, and the results thus represent a laterally averaged chemical composition. Pass energy of 50 eV was used for the survey scan.

Imaging XPS on template-stripped Ti/Au pattern samples was carried out with a PHI Quantera SXM (ULVAC-PHI, Chanhassen, MN). The X-ray source is a focused and scanned monochromatic Al Kα beam with a diameter ranging from 9 to 200 µm. The photoelectrons at an emission angle of 45° were collected and retarded with the Gauze input lens system, and were then detected by a 32-channeltron detector after passing through a spherical capacitor energy analyzer. The system was calibrated according to ISO 15472:2001 with an accuracy of ±0.1 eV. All the experiments were run at residual pressures below 5 × 10^-7 Pa. Measurements were taken on stripped Ti/Au samples with 1 mm, 30 µm, and 50 µm Ti patterns with 9 µm beam diameter and a pass energy of 140 eV in snapshot mode. The electron neutralizer was used for charge compensation.

The spectra were processed with CasaXPS software (version 2.3.15, Casa Software Ltd, Wilmslow, Cheshire, UK). Detailed mapping analyses were processed using Multi-Pak™ software (version 8.1c, ULVAC-PHI, Chanhassen, MN, USA).

8.4 Results and Discussion

8.4.1 Ti/Au Pattern vs. Au/Ag (Ag/Au) Pattern

Au/Ag or Ag/Au ultraflat surfaces were tested first, due to the known ease of the stripping step, since the adhesion between Au (or Ag) and silicon is quite poor. Therefore, the common template-stripping method can be used without an additional step of silanization of the template surfaces. However, ToF-SIMS imaging, as shown in Fig. 8.2, demonstrated a significant amount of the second deposited metal (ca. 65% of the intensity) appeared within the pattern area of the first deposited metal. This is assumed to be a result of the diffusion of the second metal into the first during the
deposition process, whereas the first metal hardly displayed diffusion into the second one.

In addition to the diffusion issue, Au and Ag surfaces exhibit similar chemical properties. Thus for the purpose of ultimately patterning with different chemistries, Au/Ag (or Ag/Au) is not a good option for further applications. Therefore, Ti/Au was then chosen for investigation. ToF-SIMS imaging (Fig. 8.3) displays a clear material contrast between the areas inside and outside the pattern. The corresponding spectra of the selected areas inside and outside pattern further confirm that there exists no detectable diffusion of one metal into the other for Ti/Au patterns.

<table>
<thead>
<tr>
<th>Peak</th>
<th>( \text{Au}^+ )</th>
<th>( \text{Ag}^{107+} )</th>
<th>( \text{Ag}^{109+} )</th>
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<td>( I_{\text{inside}}/I_{\text{outside}} )</td>
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</tbody>
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Fig. 8.2 ToF-SIMS imaging in negative mode of (a) Au/Ag pattern: mapping of Au\(^{+}\), Ag\(^{107-}\), Ag\(^{109-}\) signals, and spectra of Au\(^{+}\), Ag\(^{107-}\), Ag\(^{109-}\) signals of the selected sections; and (b) ToF-SIMS imaging of Ag/Au pattern: mapping of Au\(^{+}\), Ag\(^{107-}\), Ag\(^{109-}\) signals, and spectra of Au\(^{+}\), Ag\(^{107-}\), Ag\(^{109-}\) signals of the selected sections. Blue square represents the selected section inside the patterns for spectra and red square represents outside the patterns.

\[
\begin{array}{|c|c|c|c|}
\hline
\text{Peak} & \text{Au}^{+} & \text{Ag}^{107-} & \text{Ag}^{109-} \\
\hline
\left(\frac{I_{\text{inside}}}{I_{\text{outside}}}\right) & 0.65 \pm 0.027 \\
\hline
\end{array}
\]
Fig. 8.3 ToF-SIMS imaging of Ti/Au pattern: (a) in negative mode, mapping of Au\(^+\), Au\(_2\)\(^-\) signals, and the spectra of Au- signal for selected sections; (b) in positive mode, mapping of Ti\(^+\), TiH\(^+\) and TiO\(^+\) signals, and the spectra of Ti\(^+\) and TiH\(^+\) signal for selected sections. The purple square represents the selected sections inside the patterns for spectra and green square represents a region outside the patterns.

8.4.2 Silanization of Silicon Wafer Surfaces

For the generation of Ti/Au ultraflat surfaces, silanization of the silicon wafer is a necessary step, in order to reduce the adhesion at the metal-silicon interface. Silanes are the most frequently used reagents for the functionalization of mica, glass, silicon wafers, and some metal oxide surfaces displaying hydroxyl groups. The chemical modification is based on a self-assembly process with alkoxyilane molecules. It is generally believed that the hydroxyl groups on the surfaces attack the alkoxy groups of silane, thus forming covalent -Si-O-Si- bonding, but the detailed mechanism is still under investigation, and even the nature of this assembly process is still controversial,
with some claiming the involvement of physisorption [122]. A number of investigations have demonstrated the influence of experimental conditions on surface coverage and morphology, including silanization in the gas-phase and in solution, which can result in the formation of sub-monolayer, monolayer or even multilayers on surfaces [123-124]. For the purpose of passivation of silicon wafers to ultimately generate ultraflat Ti surfaces without residues, a high-quality silane monolayer is essential, otherwise it may result in high surface roughness or/and insufficient passivation. A densely packed, highly organized monolayer is not easy to achieve with traditional solution-phase method [124]; furthermore, many organic solvents could destroy the polymer patterns generated through photolithography process. Thus, vapor-phase silanization appears to be a better choice in general, for this process. There have been many published studies on the optimization of experimental conditions for the formation of high-quality silane monolayer from the gas phase, including the control of humidity, vapor pressure, time and so on [125-127]. As it was reported that an adsorbed water layer on freshly cleaned silicon surfaces can satisfy the requirements with respect to humidity [127], only the conditions of pressure and time were optimized in this work to achieve a high surface coverage of silanes without a concomitant increase in roughness.

Perfluorinated coatings are well known for their low surface energy and high-temperature stability. Therefore, perfluorinated silane (PFOTCS) presents a good candidate for reducing adhesion at interface of titanium and silicon. As perfluorinated coatings can also significantly reduce the adhesion between silicon wafers and polymer resists, the silanization step has to be done following the photoresist-patterning step. Hexamethyldisilazane (HMDS), is also expected to reduce the adhesion force between silicon and titanium surfaces. Besides, as it is known to function as an “adhesion promoter” between silicon wafers and polymer resists in photolithography [128], for HMDS, the silanization step can be carried out prior to photolithography, so that after the lift-off process, no additional silanization step is necessary. Therefore, both PFOTCS and HMDS could be good candidates, and the surface coverage and morphology after silanization, as well as the reduction of adhesion force and the subsequent effect on the final stripping step were investigated and compared.

For HMDS (Fig. 8.4(a)), the silanization process was conducted according to the procedure described in the literature [129]. The contact angle was measured as 87±3°, in agreement with the value in the literature [130]. AFM imaging (Fig. 8.5(a)) shows an ultraflat morphology, with an RMS roughness of 0.19±0.02 nm.
For PFTOCS (Fig. 8.4(b)), the conditions were optimized to be ca. $2 \times 10^{-1}$ mbar for 1 hour. With lower pressure (ca. $5 \times 10^{-2}$ mbar) or longer time (2.5 hours) visible aggregates were observed by AFM imaging. Under the optimal conditions, the contact angle was $107 \pm 3^\circ$, in agreement with the reported value [131], and the thickness measured by VASE was $0.89 \pm 0.05$ nm, slightly lower than the theoretical value (ca. 1.2 nm) of a full monolayer, assuming fully extended alkyl chains. The RMS roughness measured by AFM (Fig. 8.5(b)) was also low under optimal conditions ($0.21 \pm 0.03$ nm), and similar to that of HMDS silanization after a rinsing step with ultrapure water post silanization.

**Fig. 8.4** The chemical structure of the silanes used for test: (a) Hexamethyldisilazane (HMDS) and (b) 1H,1H,2H,2H-Perfluoroctyl-trichlorosilane (PFOCTS).

**Fig. 8.5** Representative AFM images of the silicon surfaces after silanization with (a) HMDS using reported protocol; (b) PFTOCS under the optimal conditions under test.
The adhesion force between the tip and the silicon surface after silanization was measured using force imaging to generate a histogram, and the results were then compared to that of the control measurement (between clean silicon wafer and tip), shown in Fig. 8.6. In agreement with the literature [132], the perfluorinated silane coating reduced the adhesion force by ca. 70% whereas HMDS reduced it by 35%. Corresponding to the reduction in the adhesion force, silanization with either HMDS or PFTOCS facilitated complete stripping of Ti/Au surfaces of ca. 1 cm*1.5 cm size with 1 mm-size Ti pattern and 1 mm distance between adjacent features (Fig. 8.7 (b)), whereas without silanization, large area of defects can be seen on Ti patterns (Fig. 8.7 (a)). Comparing silanization with HMDS and PFTOCS, (a) PFTOCS silanization could even generate ultraflat titanium surfaces of ca. 1.5 cm*1 cm size with a high rate of success (ca. 80%), whereas this value was much lower with HMDS (ca. 30%); (b) the roughness of the stripped ultraflat titanium surface from PFTOCS (RMS 0.27±0.03 nm) is slightly lower than that of HMDS (RMS 0.39±0.05 nm), which may be due to the greater temperature stability of the PFTOCS coating during the metal-deposition process. Representative AFM images of the Ti surfaces on the stripped Ti/Au pattern samples prepared using either HMDS or PFTOCS silanization are shown in Fig. 8.8.
**Fig. 8.6** Histogram images of adhesion force mapping of sample (a) clean silicon surface; (b) silicon surface after silanization with HMDS; and (c) silicon surface after silanization with PFTOCS. The Si$_3$N$_4$ cantilevers used for the measurement had a spring constant of ca. 0.6 N/m. Prior to measurements, the cantilevers were subjected to UV/ozone cleaning for 20 minutes.

**Fig. 8.7** (a) Light-microscopy (LM) image of template-stripped Ti/Au samples without silanization of silicon template, showing large area of defects of Ti pattern; (b) mosaic reconstruction of LM image showing well-stripped Ti/Au samples of ca. 1×2 cm$^2$ size prepared using silanization of the silicon template.

**Fig. 8.8** Representative AFM images of the titanium surface of the stripped Ti/Au pattern samples resulting from (a) HMDS silanization of the silicon template and (b) PFTOCS silanization of the silicon template.
8.4.3 Photolithography of 1-layer photoresists vs. bi-layer resists

The standard 1-layer photoresist was first subjected to testing for its effect on the quality of the final stripped-pattern samples. This protocol derives from previous work, which concerned the molecular patterning of surfaces [8]. However, in our technique involving metal deposition after lithography, the standard lift-off process involving immersion in a solvent, as described in the previous study, did not work quite as well, especially with small (e.g. tens-of-µm size) patterns. Sonication conditions in lift-off solution with different intensity and time were optimized to achieve complete lift-off of the photoresist together with the upper metal layer, forming a clear material contrast of patterns with structures observable by light microscopy. The final stripping process was straightforward and complete, and the stripped Ti and Au surfaces revealed atomically flat morphology, without a height difference between the chemically different regions, as determined by means of AFM imaging. However, valley-like structural defects along the entire boundary of the two materials were always observed, no matter what the pattern size and on many different batches of samples, the depth of the defect being around 3-10 nm, with a width around 100 nm (Fig. 8.7(a)). The existence of the defect was further confirmed by SEM imaging (Fig. 8.7(b)).

A bi-layer resist method was subsequently tested, which could greatly assist the lift-off process. Prior to the spin-coating of photoresist layer (S 1818), an additional layer of resist (LOR 5B) was deposited. After developing, this structure formed an “undercut” topography (Fig. 8.8), with which after metal deposition, the lift-off solvent could easily reach the LOR resist and photoresist layers, thus dissolve these layers together with the upper metal layers. In the case of the 1-layer photoresist method, the photoresist is completely covered by the metal layers, leaving no gap for the solvent to penetrate and dissolve the photoresist, which remains partially retained at the surface, especially in the case of small patterns. Although sonication could assist the removal of the photoresist layer and the metal layer above, they could be just ripped away from the metal on the substrate, leaving ragged edges or “flag” behind [133], which may result in the occurrence of the defect along the boundary of Ti/Au. Furthermore, in the case of bi-layer method, harsh conditions such as sonication could be avoided, thus reducing the damage of the deposited metal layers. As a result, AFM imaging (Fig. 8.9) shows that the Ti/Au surfaces fabricated from the bi-layer method show little evidence of the valley defect along the boundary of the two materials, which revealed similar morphology as the grain boundary of gold.
SEM imaging could also hardly detect any topographical structure along the material boundary.

(a)

(b)

**Fig. 8.7** (a) A representative AFM image of the template-stripped Ti/Au pattern surfaces resulting from the 1-layer photoresist method, showing the valley-like structural defect along the boundary of the two materials. The different morphologies of the ultraflat Ti and Au surfaces are clearly shown. A valley-like structure occurs along the boundary of these two materials; (b) SEM imaging further confirms the existence of the valley-like defects.

(a)

(b)
Fig. 8.8 (a) A schematic drawing of the bi-layer photolithography process (side view); (b) Light microscopy image exhibits the bi-layer resists of 20 µm stripe patterns after developing. The darker part indicates the two layers of resist and the lighter part besides indicates the over-hanging upper photoresist layer; (c) SEM image of the cross-section view shows the “under-cut” structure created by bi-layer method.
**Fig. 8.9** Representative AFM images of the template-stripped Ti/Au pattern surface resulting from the bi-layer resist method. The Ti part and Au part display different morphologies. Large grains and grain boundaries are visible in the Au part, but not on Ti. Little topography can be seen along the boundary of Ti/Au. (a) a 2 µm-size image and the cross-section analysis; and (b) a 1 µm-size image and the cross-section analysis.

**8.4.4 Particle Lithography**

Nanopatterns have important applications in cell biology, biosensors and biotechnology. For this purpose, particle lithography, a simple and inexpensive method to achieve ordered nanostructured masks over a relatively large area (up to mm² or even cm²), was employed for nanopatterning. Conventional methods are based on self-assembly of particles into long-range-ordered structures on substrates, and can be divided into two major categories: (1) self-assembly during solvent evaporation. Simply, monodispersed colloids (PS or silica particles in many cases) are allowed to self-organize into close-packed monolayers, driven by a combination of evaporation-induced capillary forces, gravity-driven sedimentation and Van der Waals forces. Common routes include spotting-drying and tilt-drying for large particles (several µm to sub µm), while for smaller particles, dip-drying is more appropriate [134]; and (2) self-assembly during spin-coating. The solvent flows over substrates at high shear rates during the spin-coating process, and thus particles can be densely packed on substrates rapidly, provided appropriate wettability of substrates, optimum spin speed and concentration of particle suspension. This method is more suitable for sub-µm sized rather than larger particles. Fig. 8.10 shows a PS particle monolayer prepared by the tilt-drying method for µm-size particles.
Fig. 8.10 Light microscopy image of a self-assembled monolayer of PS particles (5 µm), prepared by the tilt-drying method.

Different lift-off protocols were tested to achieve complete removal of particles. (a) sonication in different solvents (toluene, 30% ethanol+H₂O, H₂O) according to the literature, for different times until most of colloids were gone; (b) Scotch-tape stripping several times and in different directions, followed by sonication in an organic solvent to remove tape residue, and (c) step (a) together with a further cleaning step of plasma or UV/ozone or (b) together with further cleaning step of plasma or UV/ozone. However, with sonication in toluene “coffee-stain” residues were always detected by AFM imaging. Scotch tape stripping (b) also left some residues that could not be removed by further cleaning. Sonication in 30% ethanol+H₂O or H₂O did not reproducibly result in clean surfaces without residues. Correspondingly, Ti/Au patterned surfaces have never been successfully stripped using particle masks—only the Au region was stripped occasionally and in general the entire sample became stuck to the silicon template. To investigate the causes further, Au/Ag and Ag/Au samples were prepared with the same particle-lithography protocol. Occasionally, even Au/Ag samples could not be stripped. The stripped Au/Ag samples generated using only sonication to remove particles but without further cleaning by plasma or UV/ozone displayed a reversed “coffee-stain” topography, as observed by AFM imaging (Fig. 8.11 (a)). Furthermore, despite further cleaning with plasma or UV/ozone after removal of particles, the stripped
Ag/Au samples did not exhibit the reversed “coffee stain” topography, they still revealed a rough morphology and a distinct height difference between Au part and Ag part could be seen (Fig. 8.11(b)), similar to that resulting from scotch tape stripping with further UV/ozone cleaning (Fig. 8.11(c)). Thus, we assume that the failure of particle lithography was due to the “dirty” sample surfaces resulted from the evaporation of solvent [137] during self-assembly of the particle monolayer, which could not be completely cleaned with all the lift-off protocols under investigation. It could not only have a negative influence in the silanization step, leading to the formation of an inadequate passivation layer, but also it could lead to the generation of a topography difference that could possibly have an effect on the ultimate stripping step.

![Fig. 8.11](image)

**Fig. 8.11** Representative AFM images of (a) Au/Ag pattern surface generated from particle lithography without further plasma or UV/ozone cleaning during the lift-off process, displaying reversed “coffee stain” topography; (b) Au/Ag pattern surface generated from particle lithography with sonication and further UV/ozone cleaning during the lift-off process, exhibiting a rough morphology with distinct height difference between Au and Ag part; and (c) Au/Ag pattern surface generated from particle lithography with scotch tape stripping and further UV/ozone cleaning during the lift-off process, displaying reversed residue topography in the centre of each circle.

### 8.4.4 XPS vs. ToF-SIMS results

An XPS survey spectrum of the stripped Ti surfaces was acquired first (shown in Fig. 8.12). It exhibits high-intensity Ti 2p, O 1s signals, some carbon contamination, and a little F 1s signal assumed to be from the CF$_x$ group of the perfluorinated silane layer on the silicon substrate, which was reduced below 5% after UV/ozone cleaning for 1 hour. No Si 2p, Si 2s signals were detected, indicating that there were no silicon
residues from the silicon-wafer template, and the silane passivation layer can lead to a complete stripping of Ti from silicon template.

**Fig. 8.12** XPS survey spectrum acquired for template-stripped Ti surfaces. After stripping, samples were sonicated in isopropanol and subjected to UV/ozone cleaning for 1 hour prior to measurements.

XPS imaging was then carried out on Ti/Au samples of different pattern sizes: 1 mm-size Ti pattern, and 30 µm/50 µm-size Ti pattern. For patterns of 1mm Ti, different spot sizes of the beam (9 µm, 100 µm, 200 µm) were applied, and all the results indicated a clear material contrast, with no diffusion detected (Fig. 8.13 (a)). For samples of 30 µm/50 µm Ti pattern, the smallest spot size of the X-ray (9 µm) was used for the measurement. However, the Au signal was always detected in the Ti pattern area, whereas no Ti signal could be found in the Au area (Fig. 8.13(b)). This result contradicts that obtained by ToF-SIMS on the same samples, as well as that acquired for samples of the 1mm Ti pattern. It is thus proposed that the controversy is due to a minimum pattern size being required with respect to the lateral resolution of XPS. It has been reported that the beam size needs to be at least 3-4 times smaller than the structure size for effective XPS imaging [135].
Fig. 8.13  XPS mapping of Ti 1s and Au 4f signals acquired for stripped Ti/Au samples of (a) 1 mm-size Ti patterns, with no Au 4f signal detected within the Ti
pattern; and (b) 50 µm-size Ti patterns, with a little Au 4f signal detected on Ti pattern surfaces, although no Ti 2p signal was seen in the Au area.

8.4.5 Ti/Au ratio

The effect of this template-stripping method on different Ti/Au area ratios on surfaces was studied using an economical photolithography method with a black/white gradient mask designed and fabricated, as described in Spori’s previous work. A detailed description of this technique can be found in her PhD dissertation [136]. Simply, a B/W gradient mask was first generated, either (a) designed and drawn in Illustrator, and then converted into Photoshop using a “diffusion” filter. It is quasi-linear, as the distribution of dots follows displays a certain behavior upon reaching a critical density and thus the area of B/W color changes gradually (but not linearly); or (b) programmed in Matlab, with completely randomly distributed dots, whose density then changes linearly along one direction. The mask design was then printed onto a transparent film foil with a 3800-dpi laser printer. The photolithography procedure was similar to common photolithography processes, with a slightly higher UV exposure (120-150 mJ/cm²) due to a certain degree of UV adsorption of the film. In this way, gradient samples of Ti/Au density on surfaces could be generated, even with radial gradients. The photoresist pattern after developing, and correspondingly the ultimate stripped sample surface consisting of a pattern-density gradient with different area ratios (Ti/Au) are shown in Figs. 8.14(a) and 8.14(b-c), respectively.
Fig. 8.14 Light-microscopy images of (a) patterned photoresist after developing; (b) the stripped Ti/Au density gradient samples generated from the B/W gradient mask; and (c) a photograph of two stripped Ti/Au density gradients

8.5 Conclusion

In conclusion, we applied the template-stripping method to generate ultraflat surfaces consisting of composite metal patterns. While Ag/Au and Au/Ag patterns displayed significant diffusion of metals on the uppermost surface layer, ToF-SIMS and XPS showed that the stripped Ti/Au surface displayed no diffusion. Silanization conditions were tested for both HMDS and PFTOCS, in order to reduce the adhesion at the interface between titanium and the silicon template. Both silanes could be used to generate ultraflat Ti/Au patterns, with PFTOCS silanization showing a slightly better result with respect to the ease of the stripping process, and the roughness of the generated surfaces. Different relative surface-coverage ratios of Ti/Au were also achieved for this modified template-stripping method. Photolithography with either the 1-layer or the bi-layer resist method could be used to prepare large-area Ti/Au samples patterned on the mm or µm scale that, once stripped were without residues from the silicon wafers. Only the bilayer approach led to samples that were free from valley-like defects along the boundary of the two metals. Particle lithography was also used for sub-µm-to-nm patterning. However, Ti/Au samples could not be stripped from the silicon substrate, when using masks prepared from a self-assembled particle layer. The failure of particle lithography in this context is assumed to result from the contaminated surface following removal of particles under the various lift-off conditions tested.
Appendix

**Fig. A1** The B/W mask for 4’ wafer used for the demonstration of the template-stripping method on different Ti/Au ratio. Each individual B/W gradients was drawn in Illustrator (CS 4 for Mac), then converted in Photoshop using “diffusion” manner, and was then arranged in a manner in Illustrator to fit 4”-wafer size. The mask was then ready for printing. The Black dot size is around 20 μm. The sample size is of 2 cm x 1 cm, or 1.5 cm x 1 cm for gradients along one direction, and diameter of 1.5 cm for radial gradients, for the ease of stripping step.
Chapter 9

Chemical Functionalization of Ultraflat Ti/Au-Patterned Surfaces

This chapter describes the selective chemical modification of ultraflat Ti/Au-patterned surfaces and preliminary applications of the functionalized samples. The Ti and Au regions on the ultraflat sample surfaces were functionalized using catechol-and thiol-based SAM systems, respectively, to generate patterns of chemical contrast. Incorporation of a gradient-fabrication technique generated surface-chemical gradients on patterned ultraflat surfaces. ToF-SIMS, XPS, and microdroplet condensation were performed on the chemically modified ultraflat patterned surfaces.

9.1 Self-assembled Monolayers (SAMs)

Self-assembled monolayer refers to the formation of one layer of a species (e.g. molecules or particles) via their spontaneous organization into ordered structures, either from solution or the vapor phase. Commonly, these molecules consist of an anchoring group (head group) that binds to surfaces, a spacer e.g. hydrocarbon chain, and a functional group (terminal group) that determines the ultimate surface functionality [137]. The driving forces for SAM formation include the interactions between the head group and surface for binding (generally a covalent bond), as well as the Van der Waals forces between the adjacent hydrocarbon chains for array ordering [138]. Fig. 9.1 shows a schematic drawing of a typical SAM system [137].

SAM systems have been extensively studied and also applied as model systems in fundamental studies for the last few decades, thanks to their simplicity and versatility. The first SAM system was reported by Zisman in 1946, and consisted of alkylsilanes on glass surfaces [139]. Other SAM systems were then gradually established for many different materials surfaces, such as alkanethiol-Au, and catechol-TiO₂ [140, 141].
Fig. 9.1 A schematic drawing of a typical SAM system consisting of surface-active head group, spacer chain and surface terminal group, and the ordered molecule arrays with a specific tilt angle $\alpha$ due to the interchain interactions [137].

9.1.1 Alkanethiol

Alkanethiols are the most commonly used molecules to form SAMs on gold and other noble metal surfaces. For instance, it binds to gold surface via a thiolate, forming a Au-S bond, of which the nature is still controversial [142], and arranges into well-ordered arrays with a typical tilt angle of 30° to the surface normal, so as to maximize the lateral Van der Waals interactions [143]. Regarding the adsorption kinetics, alkanethiols are often reported to adsorb in two steps: (1) the fast chemisorption step reaching ca. 80% of monolayer coverage but with a low degree of order; and (2) the rearrangement of alkane chains into an ordered array, leading to the adsorption of more molecules to achieve monolayer-coverage and an all-trans orientation [144]. A Langmuir-type adsorption isotherm and slightly adjusted Langmuir-type isotherm have been often reported to fit the adsorption kinetics of alkanethiol [145-146]. The alkanethiol molecule used in our experiments is hydroxyl-terminated alkanethiol, 11-mercapto-1-undecanol, whose chemical structure is shown in Fig. 9.2 (a), and which can generate a hydrophilic surface on a gold substrate.

9.1.2 Catechol

Catechol (1,2-dihydroxybenzene) and its derivatives are surface-chemical modifiers that are found in nature. It was found that mussel adhesion to rocks involved certain catechol derivatives, i.e. amino acid-3-(3,4-dihydroxypheneyl)-L-alanine (L-DOPA), which was found in the adhesive proteins that mussels secrete [147]. This indicates
the capabilities of catechol for anchorage and adhesion. Catechol is known to chelate certain metal ions on surfaces via the two hydroxyl groups in adjacent ring positions, in monodentate and/or bidentate coordination mode, depending on system conditions [148-149]. The detailed binding mechanism is still of fundamental research interest, regarding the material of substrates and other influencing factors, e.g. pH of solution, temperature, salt concentration and so on. The strong affinity of catechol and its derivatives for a large variety of materials surfaces (e.g. metal oxides, metals, polymers, etc.) has been reported and known for many decades, and has attracted growing interests recently as a means to surface-chemical functionalization, in particular for metal oxide surfaces (i.e. TiO₂, Fe₂O₃, Cr₂O₃, MnO₂, etc.) [148-151]. The catechol molecule used in our experiments was fluorinated nitroDOPA, which, when adsorbed on surfaces, renders them hydrophobic. The chemical structure is shown in Fig. 9.2(b).

![Chemical structure](image)

**Fig. 9.2** The SAM molecules used for selective functionalization of Ti/Au ultraflat surfaces (a) 11-mercaptop-1-undecanol (MU); and (b) perfluoro-alkyl-nitrodopamine (PFAND).

### 9.2 Experimental

#### 9.2.1 Materials

11-mercaptop-1-undecanol was purchased from Sigma-Aldrich Chemicals (Karlsruhe, Germany). Perfluoro-alkyl-nitrodopamine was obtained from SuSoS AG (Duebendorf, Switzerland). All solvents were HPLC grade and obtained from Sigma-Aldrich (Buchs, Switzerland) unless otherwise mentioned.
9.2.2 Preparation Procedure

Surface Cleaning

The template-stripped Ti/Au pattern samples were sonicated in ethanol for 2 min to remove small particles generated during mechanical stripping process. Prior to chemical functionalization, samples were subjected to UV/ozone cleaning for 1 hr, immersed in ethanol for 10 min and then sonicated for 2 min to reduce oxidized gold species (AuO\textsubscript{x}) on the sample surfaces, and finally rinsed with MilliQ water. This cleaning step could reduce the fluorine contamination from the silane layer on silicon surface to less than 5%, as measured with XPS.

Chemical Patterning

For homogeneous chemical coatings, the cleaned samples were immersed in a mixed solution of MU and PFAND (v/v 2:1 isopropanol: H\textsubscript{2}O containing 0.05 mM MU and 1 mM PFAND) for 1hr. The solvent was chosen according to the established protocol for PFAND adsorption on TiO\textsubscript{2} surfaces, which was also applicable for alkanethiol adsorption on gold. Afterwards, samples were rinsed with isopropanol, sonicated for 1 min to get rid of physisorbed species, rinsed with fresh isopropanol, and finally blown dry in N\textsubscript{2}.

Surface-Chemical Gradients

Surface-chemical gradients on Ti/Au samples were generated by means of a gradual dipping process, with a computer-controlled nonlinear motion drive, as described before [119].

Firstly, the adsorption kinetics of PFAND solution (1 mM in 2:1(v/v) isopropanol/H\textsubscript{2}O) on bare Ti samples was measured. Individual Ti substrates were immersed for different lengths of time, and the static contact angle immediately measured, to yield the adsorption kinetics. Afterwards, different concentrations of MU solution were tested using 2 cm-long gold substrates with the dipping program generated from the adsorption kinetics of PFAND on Ti. Static contact angle was measured every 3 mm along functionalized gold samples. The linearly changing contact angle thus corresponds to the appropriate concentration of alkanethiol. Thus both the linearly varying MU gradients on Au regions and PFAND gradients on the Ti regions of cleaned Ti/Au samples can be generated in a dipping step in a mixture...
solution containing MU and PFAND (2:1(v/v) isopropanol/H₂O containing 0.05 mM MU and 1 mM PFAND).

9.2.3 Characterization

ToF-SIMS

ToF-SIMS measurements were carried out with a ToF.SIMS⁵ spectrometer (ION-TOF GmbH, Munster, Germany) in the mass range 0-1000 m/z. Both spectra and imaging acquisitions were done with Bi⁺⁺ primary ions, energy of 25 kV, at a current of ca. 0.3 pA for spectra and ca. 0.05 pA for imaging. All spectra were acquired with high mass resolution (≥5000). The imaging was carried out with the so-called burst alignment mode, providing good lateral resolution typically around 300 nm and unit mass resolution in a reasonable acquisition time. All surfaces were scanned with an ion dose density of 5*10¹²/cm² across 200*200 µm² area for chemically functionalized Ti/Au samples. For each sample, three positions were randomly chosen. At least 2 samples from each batch and at least two different batches were measured. For chemical-gradient samples, a position list containing coordinates of positions every 2 mm along the sample was generated with the z coordinate of each position corrected for focusing, thus measurements could be automatically done for the entire gradient samples. In order to save time, only large-patterned (1 mm Ti features) samples were measured, so that the lateral resolution of the spectra mode was sufficient. All the data were processed and analyzed with IonSpec and IonImage software (ION-TOF, version 4.1). Negative spectra were calibrated using the C-, CH-, C₂H- and Au²⁻ peaks, and positive spectra using the C⁺, CH⁺, CH₃⁺, C₂H⁺, and C₃H₄⁺ peaks.

µDD

Microdroplet condensation was measured on Ti/Au samples with both homogenous chemical functionalization and surface chemical gradients. Samples were mounted on a cold stage, which was cooled down by an ice-water mixture. The condensation was observed and captured by a CCD-camera-coupled light microscope (Axio IMAGER M1m, Zeiss, Oberkochen, Germany). For gradient samples, images were taken every 2 mm along gradients with x10 magnification.

XPS

Imaging XPS on chemically functionalized Ti/Au pattern samples was carried out
with a PHI Quantera SXM (ULVAC-PHI, Chanhassen, MN). The X-ray source is a focused and scanned monochromatic Al Kα beam with a diameter ranges from 9 to 200 µm. Photoelectrons at an emission angle of 45° were collected and retarded with the Gauze input lens system, and were then detected by a 32-channel detector after passing through a spherical capacitor energy analyzer. The system was calibrated according to ISO 15472:2001 with an accuracy of ±0.1 eV. All the experiments were run at residual pressures below $5 \times 10^{-7}$ Pa. Measurements were taken on functionalized Ti/Au samples of 1 mm Ti pattern with an X-ray beam of 9 µm size, and a pass energy of 140 eV in snapshot mode. An electron neutralizer was used for charge compensation.

9.3 Results and Discussion

9.3.1 Chemical Patterning

The homogeneous chemical modification of Ti/Au patterns with alkanethiol-based chemistry and catechol-based chemistry was characterized with μDD and ToF-SIMS. As adsorbed MU displayed hydrophilic behavior on the Au surface, and PFAND generated a hydrophobic coating on the Ti surface, the contrast of wettability could be clearly revealed with MDD characterization. Fig. 9.3 shows a qualitative characterization of the Ti and Au regions, following simultaneous, independent functionalization in a mixed MU/PFAND solution—fewer, larger water microdroplets were condensed on the Au region, while a larger number of smaller droplets can be seen on the Ti region.
Fig. 9.3 Light-microscopy image at x4 magnification of microdroplet condensation on stripped Ti/Au samples after immersion in a mixed solution of MU/PFAND. Fewer, larger water microdroplets were condensed on the Au region (yellow), while a larger number of smaller droplets can be seen on the Ti region (pink). This indicates hydrophilic behavior of the MU-functionalized Au and hydrophobic behavior of the PFAND-functionalized Ti after simultaneous treatment in a mixed solution of MU/PFAND.

The selectivity of alkanethiol-based chemistry for Au and catechol-based chemistry for Ti on Ti/Au pattern samples was further confirmed by the results of ToF-SIMS imaging of Ti/Au micropatterns subjected to the same functionalization. ToF-SIMS is known for its very high surface sensitivity. Characteristic fragments of MU and PFAND were first recognized in the spectra mode for homogeneous Au and Ti samples. Afterwards, imaging ToF-SIMS was performed and mapping of these characteristic fragments could be carried out for Ti/Au micropatterns, as shown in Fig. 9.4. It clearly displays that in the negative mode, the distribution of HS\(^-\), S\(^-\) species, which are characteristic peaks of alkanethiol MU, exactly follows that of Au\(^-\), and the distribution of F\(^-\) in the negative mode and the CF\(^+\) in the positive mode, which are characteristic peaks of PFAND, follows that of Ti\(^+\) signal. These data of ToF-SIMS imaging strongly correlates to the MDD results, that is, the independent modification of MU for Au part and PFAND for Ti part in a mixed solution without interference of the existence of either of the chemicals. The measurements by the means of MDD and ToF-SIMS illustrated that the chemical functionalization of Ti/Au pattern samples could be achieved by a single immersion step in a mixed solution of alkanethiol and catechol, which is facile and cost-effective.

The reusability of template-stripped Ti/Au samples with respect to chemical modification with alkanethiol-based and catechol-based chemistry was qualitatively demonstrated by μDD measurements on patterned samples after initial immersion in a MU/PFAND mixed solution (Fig. 9.5(a)), and subsequent treatment with UV/ozone and immersion into a mixed solution of 1-undecanethiol/nitrodopamine (Fig. 9.5(b)). As was expected, MU/PFAND generated hydrophilic/hydrophobic functionalization of Au/Ti, while 1-undecanethiol/nitrodopamine led to hydrophobic/ hydrophilic functionalization of Au/Ti. Therefore, in Fig. 9.5(a), larger and fewer water microdroplets are visible on the Au region, while smaller and a greater number of droplets were to be seen in the Ti region, whereas Fig. 9.5(b) exhibits the opposite behavior.
Fig. 9.4 ToF-SIMS imaging of chemically modified stripped Ti/Au samples after immersion in a mixed solution of MU/PFAND. Ti features are 30 µm in diameter, and an area of 200 x 200 µm² was randomly chosen for measurements. Characteristic fragment mapping of Au⁻, S⁻, HS⁻, F⁻, Ti⁺ and CF⁺ is shown here.

(a) (b)

Fig. 9.5 Light-microscopy image at x4 magnification of microdroplet condensation on (a) stripped Ti/Au samples after immersion in a mixed solution of MU/PFAND, and (b) the same sample after UV/ozone cleaning for 1 hr, followed by immersion in a mixed solution of 1-undecanethiol and nitrodopamine. This illustrates the reusability
of template-stripped Ti/Au samples regarding chemical modification with alkanethiol-based and catechol-based chemistry.

9.3.2 Surface-chemical Gradients

9.3.2.1 Adsorption Kinetics

Adsorption kinetics of PFAND were first measured by the means of contact-angle measurements of individual samples immersed for different times. The concentration of 1 mM in isopropanol/water mixture (2:1, v/v) was chosen, as it can generate hydrophobic coatings (CA 89±3°) within an appropriate time length (ca. 40 min), but only a CA of 64±2° and 75±3° with 0.25 mM and 0.5 mM PFAND solution, respectively. Afterwards, the adsorption kinetics of 1 mM PFAND in isopropanol/water mixture (2:1, v/v) at 25 °C was monitored, shown in Fig. 9.6, so as to generate the dipping program for the preparation of linear PFAND gradients on Ti samples.

![Graph showing adsorption kinetics](image)

**Fig. 9.6** Adsorption kinetics of 1 mM PFAND in isopropanol/water mixture (2:1, v/v) at 25 °C, as indicated by static-contact-angle measurements of individual samples immersed for different lengths of time.

Two different concentrations of MU solution, 0.01 mM and 0.05 mM were then tested for the generation of a MU surface-concentration gradient on Au with the same dipping programme generated from the adsorption kinetics of PFAND on Ti. Fig. 9.7 clearly shows that, with 0.01 mM of MU, the CA value at the beginning of the gradients (the first 5 positions) remained almost the same (around 70°), indicating the need for a higher concentration. Therefore, a higher concentration of 0.05 mM of MU
was then tested, with which it generated a linear change of static contact angle along the sample. The linearly changing contact angle thus corresponds to the appropriate concentration of alkanethiol, which is 0.05 mM in 2:1 (v/v) isopropanol/water.

Fig. 9.7 Static-contact-angle measurements of Au samples, gradually dipped into 0.01 mM or 0.05 mM MU solution (MU in 2:1 (v/v) isopropanol/water) with the same dipping programme used for 1 mM PFAND in 2:1 (v/v) isopropanol/water. Measurements were performed every 3 mm along the functionalized Au samples.

9.3.2.2 Characterization of Gradients on Homogeneous Substrate

Thus, by using the same dipping programme into the mixture solution of MU and PFAND, it should be possible to generate a linear surface-density gradient of PFAND on Ti, and a linear gradient of MU on Au, respectively, which was confirmed by the results shown in Fig. 9.8. The $R^2$ of the linear curve fit was 0.9901 for the PFAND gradient, and 0.9827 for MU. It illustrated that the presence of either MU or PFAND does not significantly affect the adsorption kinetics of the other.
Fig. 9.8 Static contact angle measurements of PFAND surface-density gradient on Ti substrates generated from a gradual dipping process into a mixture of 1 mM PFAND and 0.05 mM MU in 2:1 (v/v) isopropanol/water, and MU surface-density gradient on Au generated from a gradual dipping process into the mixture solution of the same composition and concentration. Measurements were done every 3 mm along the samples.

ToF-SIMS spectra were acquired on homogenous chemical coatings on homogeneous metal substrates, prior to carrying out measurements on surface-chemical gradients on Ti/Au substrates. For alkanethiols on Au, there have been a few reports of ToF-SIMS results and data analyses, including on samples of different surface coverage, which are relevant to our system [152]. For PFAND, to our knowledge, there has been no report on ToF-SIMS characterization to date. Thus, ToF-SIMS spectra along PFAND gradients on bare Ti substrates were obtained first, and the results in the m/z range of 0-200 were displayed in Figs. 9.9 and 9.10. The positively charged characteristic clusters of C$_x$F$_y$\(^+\) were recognized in Fig. 9.9, and the intensity of these clusters changes linearly along the gradient samples, as shown in Fig. 9.10.
Fig. 9.9 ToF-SIMS spectra of positively charged clusters of PFAND homogenous coating on Ti substrates, displayed in a m/z range of 0-200. Characteristic peaks of $\text{C}_x\text{F}_y^+$ are shown and labeled.

Fig. 9.10 The intensity of characteristic peaks of $\text{C}_x\text{F}_y^+$ for a PFAND surface-density gradient on a Ti substrate, measured in ToF-SIMS spectra mode. Measurements were carried out every 2 mm along 1-cm-long gradient samples. A linear fit for the measured species was performed.
9.3.2.3 Characterization of Gradients on Ti/Au substrates

The characterization of surface-chemical gradients on stripped Ti/Au pattern samples was carried out with microdroplet condensation and ToF-SIMS measurements, whose results are shown in Fig. 9.11 and 9.12, respectively. In Fig. 9.11, while the water droplets gradually became larger on the Ti features along the gradient samples, the water droplets, in contrast, gradually became smaller on the Au regions. Consistent with this, ToF-SIMS mapping of the characteristic fragments of MU and PFAND shows that while the intensity of C₃F₇ gradually decreased, the intensity of Au₂S⁺ increased. ToF-SIMS along with MDD measurements qualitatively and semi-quantitatively demonstrated the fabrication of bidirectional surface-chemical gradients on Ti/Au pattern surfaces.
Fig. 9.11 Microdroplet condensation of surface-chemical gradients prepared from stripped Ti/Au samples, gradually dipped into a mixture of 1 mM PFAND and 0.05 mM MU in 2:1 (v/v) isopropanol/water. The Ti features are 50 µm in diameter. Measurements were carried out every 2 mm along 1-cm-long gradient samples.
Fig. 9.12 ToF-SIMS fragment mapping for bidirectional surface-chemical gradients prepared from stripped Ti/Au samples, gradually dipped into a mixture of 1 mM PFAND and 0.05 mM MU in 2:1 (v/v) isopropanol/water. The Ti pattern is of 1 mm width, thus the measurements could be done in spectra mode, whose lateral resolution would be sufficient. Mapping of fragments \( \text{Au}^+ \), \( \text{Au}_2\text{S}^+ \), \( \text{Ti}^+ \), \( \text{C}_x\text{F}_y^+ \) are displayed. Measurements were carried out every 2 mm along 1-cm-long gradient samples.
9.4 Conclusion

To summarize, the ultraflat Ti/Au pattern surfaces generated from the template-stripping method can be chemically modified by means of reactions of Au and Ti with alkanethiol- and catechol-based species, respectively. Characterizations by ToF-SIMS and microdroplet condensation indicated that the Ti and Au regions were independently functionalized with a single immersion step in a solution mixture containing PFAND and MU. The reusability of the stripped Ti/Au substrates for chemical modification with alkanethiol and catechol chemistry was also confirmed by microdroplet condensation measurements. Furthermore, by careful determination of adsorption kinetics, independent catechol and alkanethiol gradients could be generated on the Ti and Au regions, respectively, by means of a single dipping process into a mixture of adsorbates.
This chapter contains the conclusions and outlook section of Project 2, summarizing the work and results in chapter 8-9, showing preliminary results of the applications of the ultraflat Ti/Au pattern substrates, and other ideas for future directions.

10.1 Conclusions

A template-stripping method was used to generate ultraflat patterned surfaces consisting of composite metal patterns—in our experiments consisting of Au/Ag and Ti/Au. This is the first report on the fabrication of ultraflat Ti/Au patterned surfaces, which can be several cm in size, robust, and without residues from the silicon substrates. The generated Ti/Au surface was characterized by means of ToF-SIMS and XPS, showing no diffusion of metals on the uppermost layer of surfaces, in contrast to the behavior of Au/Ag. To generate Ti/Au patterns, a silanization step for the silicon wafer substrate is necessary to reduce the adhesion between Ti and the Si substrate. Silanization with either HMDS or PFTOCS could enable the stripping of ultraflat Ti/Au patterned surfaces, with PFTOCS showing a slightly better result with respect to the ease of stripping process, and the roughness of the generated Ti surfaces. Photolithography was used to generate micropatterned surfaces of Ti/Au. By means of a bi-layer resist method, the valley-like defects along the Ti/Au boundary could be removed, generating ultraflat Ti/Au surfaces without detectable topography at the interface of the two materials, as evidenced by AFM and SEM. Surface-density gradients of Ti dots on Au could also be prepared for this modified template-stripping method by using a photolithographic mask consisting of black dots of gradually changing density printed on a film foil. Although photolithography was successfully applied to this template-stripping method, there were problems when using particle lithography for the generation of sub-µm-to-nm Ti/Au patterns, which may be due to the lack of cleanliness and flatness of the silicon surface after lifting-off particles.
We also showed that the ultraflat Ti/Au patterned surfaces prepared with this template-stripping method could be simultaneously functionalized by means of alkanethiol- and catechol-based chemistries. ToF-SIMS and microdroplet-condensation measurements demonstrated that the part and Au regions could be independently grafted with PFAND and MU in a single immersion step in a mixed solution of these two types of SAM-forming molecules. The functionalized Ti/Au substrates could be reused after being subjected to a cleaning step by UV/ozone, as shown by microdroplet-condensation measurements. We were also able to generate surface-chemical gradients on Ti/Au patterns using a gradient-preparation technique based on a controlled dipping programme into the mixed solution of thiol and catechol, as shown with ToF-SIMS and microdroplet condensation.

Therefore, a preparation technique and chemical functionalization method for ultraflat Ti/Au patterns have been established. Ultraflat Ti/Au patterned surfaces could serve as a versatile platform, especially to address fundamental research questions in a cost-effective manner. A question that remains is how to make good use of the advantages of (1) the ultraflatness of the substrates, and no height difference between Ti and Au parts; (2) the independent chemical-functionalization of Ti and Au parts; and (3) surface chemical gradients generated from a single dipping process, and the properties of high-throughput screening, and driving dynamic phenomena. Section 10.2 provides some preliminary results of applications of ultraflat Ti/Au patterns and suggests some future directions.

### 10.2 Outlook

Regarding the applications, since Ti/Au pattern samples show excellent flatness, no height difference between Ti and Au regions, and affinity of Au and Ti for different SAM molecules, they could be used for AFM imaging to determine accurate values for thickness of SAM layers, as well as their morphology, friction and so on. Fig. 10.1 shows the AFM imaging result of the first test on Ti/Au patterns following immersion in alkane phosphate (C16) solution for 0.5 hour. A height difference of ca. 1.6 nm between the Ti and Au regions can be observed after functionalization, and the RMS of the Ti region increased to ca. 0.7 nm after functionalization while the RMS of Au still remained ca. 0.2-0.3 nm, indicating the selective formation of a sub-monolayer of alkane phosphate on the Ti regions. The defect along the boundary of Ti/Au part was due to the use of the 1-layer photoresist method. Thus, the ultraflat Ti/Au pattern platform could assist in measuring the thickness of macromolecular layers, such as
catechol(or phosphate)-PEG, and also monitoring the adsorption kinetics of the SAM molecules.

**Fig. 10.1** AFM imaging and cross-section analysis of Ti/Au pattern samples after immersion in alkane phosphate (C16) solution for 0.5 hour. The defect along the boundary of Ti/Au part was due to the use of the 1-layer photoresist method.

Micropatterning and nanopatterning play important roles in fundamental biological research, as well as in biotechnological applications, e.g. biosensors. Therefore ultraflat Ti/Au patterned samples could serve as a platform to study cell-material surface interactions without the influence of surface topography. Fig. 10.2 shows controlled cell localization on ultraflat Ti/Au samples with 1 mm Ti features, with the Au part functionalized with thiol-PEG (5000) to generate an antifouling coating. Besides the excellent flatness, the advantage of ultraflat Ti/Au over the previously reported SMAP pattern [9] is the lack of a height difference between the two patterned material surfaces for Ti/Au, whereas differences of tens of nanometers existed on SMAP samples. The advantage over MAPL samples [8] lies in the affinity of Ti and Au for different types of SAM molecules, whereas on MAPL samples, only one material (e.g. silicon, titanium oxide) is present, and therefore only one type of molecule could be grafted onto patterns.

Surface gradients are able to drive certain dynamic phenomena, behaving as so-called “smart materials”. Whitesides’ group first reported that water droplets could move uphill on a surface-wettability gradient [19]. This concept could also be employed for the Ti/Au substrate, to drive water droplet movement along a wettability gradient generated on a Ti strip within a Au/Ti patterned surface, for the potential
applications in open microfluidics. A schematic sketch of a potential system design is shown in Fig. 10.3 (a). It consists of a channel of Ti surface to transport water, which could be functionalized with a PFAND surface-density gradient (wettability gradient), a storage area for water at the end of the transportation channel, and a surrounding Au region functionalized with 1-undecanethiol to generate hydrophobic coating, so that the water droplet would be restricted to the Ti channel. Important parameters in the design include the width and length of the Ti channel, and the size of the storage area.

The design of our system could be compared to a related system reported in the literature [153]. The most important differences lie in that (1) there is no distinct height difference between the Au and Ti part, while in the reported system there was. This factor could also have some influence in the study of water droplet movements; and (2) there was no chemical functionalization on the two materials and also no surface gradient, only a hydrophilic Si surface embedded in the hydrophobic wax surfaces.

Template-stripped Ti/Au samples of such design of pattern structure have been fabricated using the economical mask-fabrication technique described in Chapter 8. The design of the mask for a 4” wafer was shown in Fig. 10.3 (c), and the image of the stripped samples in Fig. 10.3 (b). As the sample is large, of centimeter size, occasionally a few defects were present, which may have resulted from either the presence of dust prior to the metal-deposition step, or to some flaking during the harsh mechanical stripping step if samples were not glued well to the glass slides. However, the small defects can have a significant effect on the water droplet movement, as it is a dynamic process. Therefore, to date, too few perfect samples have been generated to carry out a systematic investigation. Preliminary results show that (1) in a horizontal position, water droplets of more than 10 µL can move (or spread in the case of bare Ti) along the channel of both bare Ti and Ti with PFAND gradient; and (2) at inclined angles of 15°, at volumes up to 20 µL, a water droplet was still able to move uphill along the channel with a PFAND gradient, whereas it could not move along the bare Ti channel. More samples with a high degree of perfection need to be prepared to ensure reproducible results and carry out a systematic study.

The movement of water droplets could be further improved if the single-component gradient of PFAND were backfilled with another component of similar chain length (such as COOH-terminated catechol) so as to minimize the hysteresis of the system, which was around 30°-40° along the PFAND gradient on Ti, measured by means of dynamic contact angle.
Fig. 10.2 FM image of human umbilical vein endothelial cells (HUVEC) seeded on Ti/Au substrates after 1 day incubation in cell culture medium containing 10% FBS. The Au patterned areas were functionalized with thiol-PEG (5000) to render them non-adsorptive to proteins. The cell nuclei were stained with DAPI (blue), and cytoskeletons were stained with fluor 488-labeled phalloidin.
Fig. 10.3 (a) A schematic sketch of a wettability gradient along a Ti channel on a Ti/Au pattern surface, for the purpose of driving water-droplet motion; (b) An example of the stripped Ti/Au patterned substrates used for driving water droplets; and (c) The mask for a 4” wafer used for the generation of the template-stripped Ti/Au substrate for water-droplet movement experiments. The entire structure was drawn in Adobe Illustrator (CS 4 for Mac), and then printed on a polymeric foil (Kodak Gen 5 GRD, EncapSulite International Ltd., Germany), which was used for photolithography. The width of the test channels was 1 mm or 0.5 mm, the length 1 cm. The circle-shape storage area for water was of 4 mm or 6 mm diameter.
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Research Experience

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(1) Preparation of surface-density gradients of polymer chains and their applications in studying and tuning protein adsorption and cell adhesion for bone implant applications (in situ measurement of adsorption kinetics of polymer onto metal oxide surfaces by OWLS, surface-gradient characterization by ellipsometry & fluorescence microscopy, protein adsorption, cell culture, cell adhesion and fluorescence staining assay, and collaboration in bacterial-adhesion assay);

(2) Development of laterally patterned ultraflat metal/metal surfaces and their functionalization with selective chemistry (spin-coating, photolithography, particle lithography, E-beam evaporation, organic synthesis & SAM formation, AFM
topography and lateral force imaging, ToF-SIMS spectroscopy and imaging, XPS spectroscopy and imaging)

2006-2008 **MSc. thesis work** on grafting NTA (nitrilotriacetic acid) on silicon surface for biosensing applications (grafting, characterization, protein enrichment, producing microarrays and FT-IR, MALDI-TOF and AFM analysis)

2005 **BSc. thesis work** on modification of porous silicon surface with undecylenic acid and NHS for protein immobilization and learning XPS, FT-IR, and SEM.

**Teaching & Supervising Experience**

2010 **Supervisor** of a Master’s student’s semester project on study of protein adsorption and cell adhesion by the use of surface gradients of bioinert polymers

2010 **Tutor** of 2 undergraduates for Forschungslabor II (Research Lab) for one semester in ETH Zurich, introducing and explaining my project and demonstrating experiments and instruments involved

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Presentations

2011 Spatial Control of Surfaces (oral presentation), Pei J, Spencer ND, Colloquium D-MATL, May 17, ETH Zurich, Switzerland.

2009 Application of PLL-g-PEG Gradients for the Investigation of Cell and Bacterial Adhesion (poster presentation), Pei J, Hall H, Rosenberg K and Spencer ND, Gordon Research Conferences on Biomaterials: Biocompatibility / Tissue Engineering, July 19-24, Holderness, NH, USA.


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