Doctoral Thesis

Molecular assembly patterning by lift-off at the micro- and nanoscale for applications in the biosciences

Author(s):
Falconnet, Didier

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MOLECULAR ASSEMBLY PATTERNING BY LIFT-OFF AT THE MICRO- AND NANOSCALE FOR APPLICATIONS IN THE BIOSCIENCES

A dissertation submitted to the
SWISS FEDERAL INSTITUTE OF TECHNOLOGY ZURICH

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presented by

DIDIER FALCONNET

Diploma in Material Sciences (EPF Lausanne) 2001
born on April 11, 1976
citizen of Vuisternens-en-Ogoz (FR)

accepted on the recommandation of

Prof. Dr. Marcus Textor, examiner
Prof. Dr. Viola Vogel, co-examiner
Prof. Dr. Jürgen Brugger, co-examiner

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Abstract

A number of applications in the biosciences such as DNA-chips, protein microarrays and cell-based sensors rely on chemically patterned surfaces. Such surfaces are essentially characterized by bio-interactive and non-interactive areas. The ability to host single cells or ensembles of few cells in well controlled surface-microenvironments has proven to be useful to study the fundamental mechanisms involved in cell-substrate interactions. Several surface cues are known to steer cellular development into a particular phenotype, namely, the type and density of cell-binding ligands, the substrate stiffness, the topography/roughness and the cell spreading. The desire to unravel the complex interplay between these different factors has created a substantial need for improved surface and interface modification tools that allow a quantitative and precise control of individual surface cues as well as their combination.

This thesis describes the development and optimization of a novel, reproducible and cost-effective patterning process named Molecular Assembly Patterning by Lift-off (MAPL). The attractive features of the MAPL technique are its abil-
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ity: to quantitatively control type and surface density of the biointeractive ligands in the adhesive patches; to elicit highly specific and quantitative interactions with the biological medium while inhibiting non-specific interactions; to control the pattern geometry/size independently of the bioligand surface density; and to produce complex pattern geometries of dimensions ranging from hundreds of microns to 100 nm.

MAPL combines a top-down approach based on photolithography and a bottom-up strategy through the self-organization of multifunctional molecules: a photoresist pattern is transferred into the desired biochemical pattern by means of spontaneous adsorption of a biologically functionalized, polycationic PEG-graft copolymer, followed by photoresist lift-off. The background surface, between the biointeractive patches, is rendered non-fouling by a simple dip-and-rinse process to form a monolayer of the corresponding non-functionalized PEG-graft polymer. Each step of the process was extensively characterized by various ultrahigh vacuum, in situ and optical microscopy surface analysis techniques (XPS, ToF-SIMS, OWLS, ELM, AFM, CLSM and SNOM). MAPL patterns are shown to have precisely controlled bioligand surface density (e.g., biotin or cell-adhesive peptide) within the patterns, and minimal non-specific adsorption of biomolecules. Note that each pattern for a given MAPL surface contains the same type and surface density of ligands. The culture, in serum-containing media, of small cell populations as well as single cells in a predefined shape and location demonstrates that MAPL is suitable in the context of studying independently the effect of ligand surface density and cell-surface contact area ("footprint"). Surfaces patterned by the MAPL technique turned out to be a versatile platform for other bioapplications as well, namely, patterning of DNA-tagged vesicles, colloidal particles and proteins. Preliminary investigations demonstrated the feasibility of using MAPL surfaces to produce protein microarrays with well-defined spot
geometries that are defined by the pattern geometry rather than the spotting process. In addition, by combining nanoimprint lithography and MAPL we were able to produce functional nanopatterns in the 100 nm range. The nano-MAPL surfaces are promising platforms for the precise arraying of single protein and vesicles and for fundamental cell-surface investigations with the aim to study and control focal complex and focal adhesion formation at the level of single ECM/integrin clusters.

MAPL is demonstrated in this work to be a technique that is robust, compatible with large batch processing and applicable to comparatively large areas such as 10 cm wafers. Furthermore, it is compatible with the requirement of the transparent substrate materials for inverted, high-resolution microscopy and optical sensing applications. These aspects make MAPL a cost-effective technique with a substantial potential for industrial applications as well as fundamental research.
Résumé

Un certain nombre d'applications issues des biosciences telles que les chips d'ADN, de protéines, ainsi que des senseurs incorporant des cellules vivantes utilisent des surfaces structurées par des motifs chimiques, qui alternent des régions biologiquement interactives avec des zones non-interactives. La capacité de cultiver des cellules, isolées ou en groupe restreint, sur des surfaces hautement contrôlées, s'est avérée utile pour l'étude de mécanismes cellulaires fondamentaux. Le développement cellulaire, caractérisé par un phénotype particulier, est guidé par diverses propriétés de surface telles que le type et la densité de ligands, la rigidité du substrat, la topographie et/ou la rugosité ainsi que l'aire de contact de la cellule avec la surface. Le désir d'élucider l'interdépendance entre ces paramètres et leurs effets sur les cellules a généré le besoin de développer des surfaces nouvelles, permettant un contrôle quantitatif sur leurs propriétés individuelles ainsi que leurs combinaisons.
Cette thèse décrit le développement et l'optimisation d'un nouveau procédé reproductible et économique, nommé "Molecular Assembly Patterning by Lift-off" (MAPL). Les aspects innovant de la technique MAPL permettent un control quantitatif du type et de la densité de surface des bioligands dans les zones bio-interactives, l'étude des interactions purement spécifiques et quantitatives avec le milieu biologique tout en empêchant des adsorptions non-spécifiques, un control de la géométrie et la taille des motifs, indépendamment de la densité de bioligands, ainsi que la production de domaines chimiques aux géométries complexes et dimensions variables, s'étendant de quelques centaines de microns à 100 nm.

MAPL combine une approche de "haut-en-bas" basée sur la photolithographie avec une stratégie de "bas-en-haut" par l'auto-organisation de molécules multifonctionnelles: les motifs transférés dans une couche mince photosensible sont convertis en motifs biochimiques par l'adsorption spontanée d'un copolymère polycationique greffé de chaînes de PEG fonctionnalisées, suivi d'une dissolution de la couche photosensible. La surface séparant les zones fonctionnalisées est rendue biologiquement inerte par un simple procédé d'adsorption et de rinçage, formant une monocouche de copolymère greffé de PEG non-fonctionnalisé. Chaque étape du processus a été caractérisée par diverses techniques d'analyse de surface dans le vide, dans l’ambiant ainsi qu’en solution (XPS, ELM, ToF-SIMS, OWLS), ainsi que par des techniques de microscopie optique (AFM, CLSM et SNOM). Les motifs produits grâce au procédé MAPL ont démontrés la capacité de contrôler précisément la densité de surface des bioligands (ex. biotines ou de peptides) à l’intérieur des zones actives, tout en éliminant l'adsorption non-spécifique d’autres molécules biologiques. Il est à noter que sur une surface MAPL donnée, chaque motif sera composé d’un même type de ligand ainsi que d’une densité de surface identique. La culture en présence de sérum de petites populations de cellules ainsi que de cellules isolées avec une forme et une position
prédéfinie démontre que ces surfaces permettent d'étudier les effets de la densité de ligands indépendamment de l'aire de contact cellule-surface ("empreinte cellulaire"). Les surfaces structurées par MAPL se sont avérées être des plate-formes expérimentales polyvalentes pour d'autres applications relatives aux biosciences, en particulier pour l'immobilisation ordonnée et contrôlée de vésicules fonctionnalisées avec des brins d'ADN, de particules colloïdales ainsi que de protéines. Des résultats préliminaires ont démontrés que l'application des surfaces MAPL pour la fabrication de rangée micrométrique de protéines permettait de produire des domaines de protéines immobilisées de grande qualité dont la géométrie est dictée par le motif de surface et non par le procédé d'impression de protéines. D'autre part, en combinant le procédé MAPL avec la technique de nano-impression, nous avons produit des motifs fonctionnels de l'ordre de 100 nm. Les surfaces structurées à cette échelle constituent un intérêt particulier pour l'immobilisation ordonnée de protéines ou de vésicules isolées ainsi que pour la recherche fondamentale sur la formation de contacts focaux à l'interface substrat-cellule.

Dans ce travail de thèse le procédé MAPL est démontré comme une technique robuste et applicable en série sur de relativement grandes surfaces tels que des wafers de 10 centimètres de diamètre. En outre, la technique s'applique à des substrats transparents, nécessaires à la microscopie inversée de haute résolution. Ces aspects font de MAPL une technique économiquement compétitive, dotée d'un potentiel non seulement pour des applications industrielles, mais aussi pour la recherche fondamentale.
CHAPTER 1

Introduction and scientific background

Materials employed in biomedical technology are increasingly being designed to elicit specific, desirable interactions with their biological surroundings. Materials with innovative properties are emerging thanks to the joint efforts of scientists from chemistry, material science and biology. This interdisciplinary approach describes well the relatively young field of bioengineering, where the contribution of a wide array of expertise is needed to create new tools dedicated to a better understanding of the exciting, but highly complex world of biological mechanisms. Nowadays, an impressive amount of commercial products are derived from biotechnology research. They reflect the great need and potential for new development and intellectual progress. To put in perspective the width of these multidisciplinary research efforts, we gathered a short list of areas where biomaterials have played an enormous role in the past years: medical devices, such as load-bearing implants (hip, knee, teeth), heart valves or vascular stents; drug delivery devices to precisely target the right molecule with a spatio-temporal control; tissue engineering where, synthetic scaffold should guide the morphogenesis of tissues in vitro. Researchers have been able to produce tissues of cornea or living heart muscle cells and implant them with success into patients. Also, many domains, not necessarily related to implants, largely benefit from the
multidisciplinary approach by combining, for example, cell-biology and microfabrication. Among the most important are biosensors (DNA (genomics) and protein (proteomics) microarrays); the emerging field of bio-microelectromechanical (BioMEMS) systems; the drug discovery process based on cell arrays (cellomics) and the fundamental research on cell-surface interactions (e.g., to control the cell phenotype). These fields have seen their development hand-in-hand with the development of chemically micropatterned surfaces. Chemical patterned surfaces are essentially characterized by geometrically and spatially defined areas of biological interactiveness in a non-interactive background. When such islands are organized in a regular array format, each pattern can be addressed individually and repeatedly in order to perform a single measurement or to study dynamic processes. The next section will give an introduction on the importance of chemical micropatterns in biotechnology and in the biosciences.

## 1.1 The role of chemical patterns in the biosciences

The ability to create patterned surfaces with locally specific biological properties has opened tremendous opportunities. The intense competitiveness at the national and international level greatly stimulates the development of novel methods and techniques that are essential components in modern biotechnology. Probably the most obvious and popular technological achievement generated by these efforts is the DNA microarray chip. Research in the area of genomics in the past 20 years paved the way to the development of patterns in a high density array format. Such arrays are composed of thousands of spots of different oligonucleotides thus allowing complex mixtures of RNA and DNA to be interrogated in a parallel and quantitative fashion. DNA arrays are used for many different
Introduction and scientific background

purposes, most prominently to measure levels of gene expression (messenger RNA abundance) for tens of thousands of genes simultaneously. The microarray format forms a revolutionary new generation of assays because of its unequaled capabilities of massive parallel processing and the minute amounts of analyte required.

The tremendous advantages of such chemically patterned surfaces are now widely acknowledged in other fields of biosciences as well. Great attention is currently given to functional genomics (proteomics) in order to manufacture protein microarrays suitable for reproducible and quantitative bioassays. Protein microarrays are likely to revolutionize medicine since most drug targets are proteins. Furthermore, the analysis of protein expression will bring light to the molecular basis of disease. However, the technological challenges to be solved to produce protein or polysaccharide (glycomics) microarrays with comparable quality to DNA-chips are far more important than for the latter. While DNA are fairly stable molecules, proteins suffer from rapid loss of activity if the environment (surface and media) is not suitable. The amount of different proteins (including conformational changes and post-transcriptional modifications) renders the task even more daunting if thousands of different proteins are to be immobilized on a single substrate. These questions are addressed in more details in chapter 7.

The relatively young areas of cell-based biosensors (cellomics for drug discovery or environmental purposes) as well as the growing research interests focusing on fundamentals of cell-surface interactions are both evolving at a rapid pace thanks to the technological progresses in surface patterning at the micro and the nanometer scale. Cell-based biosensors, in a microarray format of selectively localized living cells, are useful for high throughput screening/identification of lead compounds. Cells containing engineered fluorescent probing targets
analyzed at the inter-cellular, sub-cellular and molecular levels with automated read-out tools will potentially optimize the selection of lead compounds in the early stage of the drug discovery process.\textsuperscript{15,17} Excellent reviews on these topics can be found elsewhere.\textsuperscript{18-20}

The behavior of living cells is dictated by numerous factors of the local microenvironment. Beginning of the twentieths century gatherings of medical scientists were animated by the potential of cultivating whole living cells outside of the body.\textsuperscript{21} The birth of tissue engineering has been made possible by the development of culture growth media supplemented with a variety of soluble compounds (essential nutriments, antibiotics, etc). While soluble factors (from the media or produced by the cells) are established to be crucial for the behavior of cell survival,\textsuperscript{22} other parameters related to the surface properties have been shown to dramatically affect the behavior of surface-adherent cells in terms of attachment shape/spreading and phenotype expression. Surface factors affecting cellular development include the type of bioligands (proteins, peptides, growth factors, etc) available to the adherent cells; the surface density and the spatial distribution of the bioligands; the substrate stiffness and topography and, consequently the cell shape/spreading (see Section 1.3).

Independently of the application, all chemically patterned surfaces share a number of common issues. The positioning and immobilization of the bioactive molecules at the micro- or nanometer level, requires clever patterning strategies. A vast body of approaches are available (micropatterning: Section 1.5; nanopatterning: Section 8.1), spanning from high-resolution spotting to photolithography and surface probes techniques such as dip-pen nanolithography. Also, inhibiting non-specific adsorption from the biological environment is a key requirement for any assay since the aim is to locally elicit specific, desired interactions. Progresses in surface chemistry led to synthesis of novel molecules that are bio-
logically inert or that significantly reduce the non-specific adsorption of proteins at surfaces (Section 1.4 briefly reviews some non-fouling chemistries).

The following section provides the reader with some basic information on cells and cell adhesion.

### 1.2 Basics on Cell Adhesion

Adherent cells require a substrate to attach to in order to be viable. Such anchorage-dependent cells have four major classes of cell-adhesion receptors embedded in their plasmic membrane, namely the cadherins (cell-cell adhesion), the immunoglobulin superfamily, the selectins and the integrins. Integrins, unlike all others, are heterodimers constituted by alpha and beta subunits. They are the best understood cell-adhesion receptors since their discovery in the late 1980. Integrins and their ligands play key roles in cell development and are at the heart of many human diseases, genetic, autoimmune and others. In mammals, there are genes for eighteen α and eight β integrins; many α–β combinations fail to occur but at least two dozen are well defined. Most integrins are predominantly or exclusively receptors for specific domains of extracellular matrix (ECM) proteins such as fibronectins, laminins, vitronectins, fibrinogen and collagens. Approximately a third of the integrins bind to the well known RGD tri-peptide sequence. Integrin-mediated adhesion is a highly regulated process involving receptor activation and mechanical coupling to extracellular ligands. In addition to their role as mechanical linkers they also function as biochemical signaling hubs for many regulatory pathways. Bound receptors rapidly associate with the actin cytoskeleton and cluster together to form focal adhesions, discrete complexes that contain structural proteins such as vinculin, talin, and R-actinin as well as signaling molecules, including FAK, Src, and pax-
Focal adhesions are central elements in the adhesion process, functioning as structural links between the cytoskeleton and the extracellular matrix but also triggering signaling pathways that direct cell proliferation and differentiation. Figure 1.1 illustrates in a simple manner the complexity of these systems. We refer the reader to the review of Schwartz et al. and to the review of Siebers et al. centered on the role of integrins in bone engineering.

![Figure 1.1 Schematic diagram of a focal adhesion with structural and signaling molecules. Reproduced from Alenghat and Ingber.](image)

By taking some distance from the focal adhesions and by looking at the cell as a whole entity it is now widely accepted that cell proliferation, differentiation, migration and cell survival are governed by a number of parameters related to the surface in addition to the soluble factors:

1) **The type of ligands** (proteins/peptides) available to the cell-receptors will determine **which receptors will be recruited** and consequently, which signaling cascades will be activated.
ii) It has been shown that the **surface density** of a given ligand influences the cell response. The density of cell-binding domains of ECM proteins (epitopes) available to the cell is initially controlled by the underlying surface chemistry. For example, Garcia and coworkers have shown that fibronectin (Fn) adsorbs differently on differently terminated alkanethiols on gold, namely with the adsorbed mass increasing in the order: -OH<<-COOH<<-CH₃<<-NH₂. The authors concluded that hydrophilicity and surface charges were the major players in Fn adsorption and the subsequent conformational changes. Similarly, variations in Fn adsorbed masses of a factor of three were found between polystyrene (bacterial or tissue culture) substrates and collagen membranes. Whenever differences in Fn adsorption occurred, marked **differences in integrins recruitment** were observed. Keselowsky *et al.* developed a quantitative method for analysis of integrin binding based on crosslinking and extraction. They were able to show, for human Fn, that both heterodimers, α₅β₁ and α₅β₃, were activated for focal adhesion formation. On a synthetic peptide containing only the RGD (Arg-Gly-Asp) epitope they observed that α₅β₁ was detectable only at a background level while α₅β₃ was present in significantly higher amounts. They concluded that both integrins bind to the RGD motive in the 10th type III repeat of FN, but that α₅β₁ binding also requires the PHSRN synergy site in the 9th type III repeat. This illustrates the difficulty to use proteins as a basis to design well defined model surfaces since the **surface density of protein can be controlled but not necessarily the amount of ligands available to the cell**. This is especially true with a large protein such as fibronectin. This partially explains the success of synthetic peptides in the field of biomaterials. Immobilizing peptides on a surface is a relatively easy approach to control the surface density of a specific ligand and its presentation towards the media. The dream behind the synthetic peptide approach is to be able to bind, to the biomaterial surface, specific cells
that would be beneficial for wound healing or bone growth, while inhibiting the adhesion of immune cells.\textsuperscript{41} Today, no publication demonstrated in a convincing way that this approach is successful \textit{in vivo}. However, many \textit{in vitro} studies on peptide modified surfaces have contributed to improved understanding of cell-surface mechanisms, particularly in the area of bone regeneration. Rezania \textit{et al.} showed that \textbf{mineralization was significantly increased} when rat calvaria osteoblast-like cells where cultivated on surfaces modified with RGD peptides with a density higher than \textasciitilde{}0.62 pmol/cm\textsuperscript{2} of RGD compared to surfaces coated with adsorbed serum proteins.\textsuperscript{42} Also, Healy and colleagues demonstrated that by plating the same cells on a biomimetic surface composed of two types of peptides (RGD and heparin-binding domain) the formation of mineralized ECM was even further increased compared to surfaces that contained RGD or heparin-binding peptides only.\textsuperscript{43} Although short synthetic peptides are far less complex than native proteins, their conformation can still have a major influence on the cell response. For example, fibroblasts have a \textbf{higher affinity to cyclic RGD than linear RGD}; the cyclic conformation exhibiting a two fold increase in cell attachment rate at the same peptide density.\textsuperscript{44} Furthermore, \textbf{the number of focal adhesions (FA) was doubled on surfaces with immobilized cyclic RGD in comparison to linear RGD}. Griffith and coworkers established that the nanoscale clustering of peptides influences cell migration and spreading differently in comparison to randomly separated ligands;\textsuperscript{45,46} for the same average YGRGD density, a significantly higher fraction of cells exhibited well formed stress fibers and focal contacts when the ligands were presented in a clustered form (n=9) rather than a homogeneous ligand presentation (n=1). \textbf{Peptides are also limited by their synthetic nature} and are not able to initiate the numerous intra-cellular signals that a native "bed" of proteins would do.\textsuperscript{47} Precisely, for that reason, peptides are useful molecules for model surfaces because they enable the indepen-
dent investigation of several surface cues as well as potential applications *in vivo*. In conclusion, the use of peptides versus proteins must be motivated by the specific questions and objectives to be addressed.

iii) A third aspect of cell adhesion that was largely neglected until recently is dealing with the **substrate compliance**. This aspect was recently reviewed by Wong *et al.* Substrate compliance is especially important for contractile cells such as fully differentiated smooth (SMC) or skeletal muscle cells which ultimately transmit acto-myosin contractions through cellular attachment. Dennis Discher and coworkers addressed this question on gels in combination with collagen surface density. They reported that 24 hours after plating the SMC had a **three times larger contact area on the stiff substrate** (collagen coated glass, $E_{\text{coll-glass}}=66$ kPa) compared to the soft gel ($E_{\text{gel}}=8$ kPa). On extremely soft gels SMC could not spread under any collagen density. Additionally, **focal adhesions and cytoskeletal organization, tend to be lost on soft gels** ($E_{\text{gel}}=1$ kPa). The mechanosensory process has been quantified by clever setups of fluorescent markers embedded in the substrate or by measuring the displacements of flexible pillars supporting the cells. Gray *et al.* were able to produce chemically homogeneous surfaces with islands of different compliance compared to the background. They showed that the **cells preferentially accumulated on the stiffer regions**. Joyce Wong and colleagues developed a technique to linearly control the stiffness of a polymeric substrate based on the combination of a microfluidics gradient generator and photopolymerization. They observed notably that the cells were significantly **more spread on the stiffer regions** and adopted a rather round morphology on the more compliant surface. Analysis of individual cells revealed that the spread cells had an organized actin network, while cells on the softer region displayed diffuse staining. Recently, Berg *et al.* published a patterning technique allowing a control of ligand density on the
adhesive islands.\textsuperscript{57} This article is the only one, to our knowledge, that presents patterns with similar capabilities as the MAPL technique presented in this thesis. Their approach is based on micro-contact printing of polyelectrolyte multilayers. By tuning the pH, they were able to control the amount of deposited ligands. They mainly observed that a majority of WT NR6 fibroblasts displayed well-formed stress fibers and FA on RGD densities of over 83’000 µm\textsuperscript{-2}. This value is higher by three orders of magnitude than what has been reported for a similar phenotype.\textsuperscript{58} They attribute these differences to the softness of the multilayers, which is unfavorable for formation of FA and stress fibers.

iv) Surface \textbf{topography and/or roughness} have been shown to play a role in the differentiation process of bone forming cells (osteoblasts). \textbf{As the surface becomes rougher, various bone cells exhibit a more differentiated phenotype} based on decreased proliferation and increased activity of alkaline phosphatase and osteocalcin production.\textsuperscript{59} Other markers for osteoblast differentiation such as PGE\textsubscript{2} (prosteoglandin) and TGF-\textbeta\textsubscript{1} (growth factor) have also been shown to increase with roughness.\textsuperscript{48,60} For further details the reader is referred to the excellent reviews of Craighead and coworkers\textsuperscript{61,62} as well as the comprehensive work of Flemming et al.\textsuperscript{63} and the "Curtis-lab".\textsuperscript{64-66}

v) Cell behavior is also dictated by \textbf{the degree of spreading} or the allowed cell-surface contact area; a cell growing on a homogeneous surface is likely to respond differently than a cell that is constraint and has limited spreading possibilities\textsuperscript{67} or that is forced to grow in a given direction. According to the available literature, the discussion about the role of cell shape in growth control was mostly initiated by Folkman and Moscano in late 1970’s.\textsuperscript{68} They found that cell shape could be varied by modifying, in a graded manner, the adhesiveness of plastic tissue culture dishes. Cell height was consistently found to be inversely proportional to the degree of spreading. But their most striking finding was that
when they sparsely plated cells on the substrate (no cell-cell contacts), with the appropriate adhesiveness to hold cells at the same height as their confluent counterparts on unmodified plastic (max. adhesiveness), levels of DNA synthesis were highly similar i.e., low as opposite to highly spread and proliferating cells. This initial result suggested that cell shape per se governs cellular behavior (e.g., differentiation) rather than the cell-cell contacts found in confluent layers. These early findings paved the way to a better understanding of the influence of cell shape as the wide body of literature available nowadays testifies. However, despite of the large amount of data available today and due to the complexity of these physiological phenomena, the community agrees that the research on cell-surface interactions is still at a very early stage and that the challenges ahead will be to understand the role of the separate actors influencing the cell phenotype and function. In an effort to summarize important results, the following section reviews what we believe to be the most relevant discoveries of the past two decades in the area of cells cultured on chemical micropatterns.

1.3 WHAT WE HAVE LEARNED IN THE PAST YEARS ON CELLS CONSTRAINED TO CHEMICAL MICROPATTERNS, A REVIEW

This section focuses on adherent-cells grown on chemical patterns from one to several hundred micrometers i.e., sub-cellular structures as well as domains to accommodate multiple cells. A review on the microfabrication techniques to produce such micropatterns can be found in Section 1.5, while advances in nanobiotechnology related to cell-surface interactions are reported in chapter 8.

The five major surface cues detailed above have been difficult to study because of the inherent difficulty to present defined spatial and temporal stimuli. In order to address these questions, new tools based upon microfabrication tech-
nology, surface chemistry and materials science are being developed to allow greater control over the spatial organization and temporal presentation of the cellular microenvironment. The present summary focuses on arrays of single cells, or few cells per island, rather than on large patterns resembling traditional cell cultures with cell-cell contacts. In fact, more than ever before, attention has been given to single cell studies rather than evaluating at the end of the cell culture a cellular response that is averaged over a large and heterogeneous population of cells. The "single-cell trend" has mainly gained attractiveness for two reasons: Firstly, the new technical possibilities such as *in situ* PCR, advanced fluorescent markers such as molecular beacons and means to manipulate single cells have become a reality. Secondly, the critics formulated by Levsky and Singer (and others) is gaining more and more support; their "warning" sounds as follows: "we understand the expression of genes in terms of a cell population as all of our information comes from samples containing millions of cells. From a complex mixture of cells, we attempt to infer the probable state of an average cell in the population. In truth, what we obtain is an averaged cell, a contrivance for representing biological knowledge beyond the limits of detection. We never know the variation among the members of the population that our methods average into a mean." Arrays of single cells, separated by cell/protein repellent molecules, are ideal tools because they allow the study of thousands of single cells in a controlled, homogeneous or heterogeneous environment. Furthermore, each cell can be addressed repeatedly at different time points via the x-y coordinates, task highly difficult on randomly growing cells.

In 1988, Watt *et al.* observed *in vitro* that the proliferation and terminal differentiation of epidermal keratinocytes was regulated by the area of contact with the substratum or now commonly referred as cell shape. When spreading was restricted and the cells remained rounded, involucrin (a marker for terminal dif-
differentiation of keratinocytes) expression was stimulated and DNA synthesis was inhibited. Control experiments showed that inhibition of DNA synthesis was not, in itself, a sufficient stimulus for involucrin synthesis. Furthermore, expression of involucrin decreased in a dose-dependent manner with increasing island size, respectively cell shape, while DNA increased with contact area. The authors conclude that restricted contact area may result in coordinate regulation of a number of different genes in a way that may mimic the program of terminal differentiation in vivo. These results can be considered as a milestone since they provided evidence for a direct role of cell shape in the absence of cell-cell interactions similar to the study of Folkman ten years earlier.

Scientists focusing on liver repair provided interesting informations on hepatocyte behavior. Primary rat hepatocytes were cultured in the presence of a hormonal defined medium on variously sized laminin-containing adhesive squares. DNA synthesis was progressively increasing with pattern area to reach a maximum in unconstraint cells. On the smallest islands (<1600 μm²) less than 3% of the cells entered S phase (DNA synthesis) and viability was not affected. Moreover, hepatocytes cultured on unpatterned substrata rapidly lost the ability to secrete high levels of albumin, a liver-specific product. In contrast, hepatocytes maintained near normal levels of albumin secretion for at least 3 days when cultured on the 40x40 μm² islands. Otsuka et al. recently reported at least one month of albumin secretion when the hepatocytes were co-cultured with underlaid endothelial cells on micropatterns.

Tissue morphogenesis requires the provision of specific spatial and temporal cues at the genetic, molecular and cellular levels. Formation of branched capillary networks (angiogenesis) is also governed by these surface cues; therefore understanding these mechanisms more precisely could help developing new forms of anti-cancer therapy. In 1997-1998, Christopher Chen and colleagues
made a significant contribution with two publications\textsuperscript{67,80} (together, cited over 900 times) reporting the behavior of human capillary endothelial cells on chemical patterns. They observed that programmed cell death (apoptosis) progressively declined when the island size was increased from 75 to 3000 μm\textsuperscript{2}, whereas DNA synthesis was concomitantly switched on as cell and nuclear spreading were promoted. 20\% cell death was recorded on 75 μm\textsuperscript{2} (diam. 10 μm) and was reduced to 11\% on 300 μm\textsuperscript{2} (diam. 20 μm). To determine if the switching between apoptosis and growth was due to cell shape or cell-ECM contact area, various sub-cellular Fn patterns, separated by non-adhesive regions, were used.\textsuperscript{27,67} On these substrates, DNA synthesis scaled directly with projected cell area and was suggest to be independent of cell-ECM contact area. Apoptosis was similarly switched off by cell spreading, even though the cell-ECM contact area remained constant under these specific conditions. They conclude that cell shape \textit{per se} appears to be the critical determinant that switches cells between life and death and between proliferation and quiescence.\textsuperscript{67}

Healy and coworkers used micropatterns to modulate the nuclei shape by controlling the shape of rat calvaria bone cells.\textsuperscript{70} They reported that, independently of the cell projected area (large or small patterns), proliferation rates decreased significantly at 1, 2 and 5 day time points when compared with unpatterned surfaces (unconstrained). Collagen I synthesis (a marker for osteoblast differentiation) was strongly coupled to the cell projected area with almost no Coll\textsuperscript{I} synthesized by either, cells on 400 μm\textsuperscript{2} patterns or unconstrained cells. Interestingly, significantly higher percentages of cells synthesized Coll\textsuperscript{II} on patterns >1600 μm\textsuperscript{2}. Coll\textsuperscript{II} synthesis was found to correlate with a metric measure of the "flatness" of the nuclei (max. nuclear area in x-y divided by nuclei height). Qualitative \textit{in situ} single cell real time PCR revealed a similar trend for another marker of osteoblast differentiation, namely osteocalcin mRNA. Based on these
results the authors conclude that the patterned cells are forced to differentiate at a significantly earlier time point than unpatterned cells and that these findings could lead to implants with stronger and quicker integration.\textsuperscript{70}

Discher and colleagues were able to fuse myoblasts patterned on collagen stripes, into myotubes.\textsuperscript{81} Additionally, they observed that myotubes displayed myosin/actin striation only on stripes with stiffness typical of normal muscle cells and not on more rigid substrates. This work is a good example of how the substrate parameters (pattern shape and substrate stiffness) can control the phenotype of a specific cell type, here muscle cells.

Recently, the behavior of human stem cells was studied with the help of micro-patterns. McBeath \textit{et al.} demonstrated that they were able to guide the commitment of multipotential human mesenchymal stem cells in either, adipocytes or osteoblasts.\textsuperscript{82} The cell shape, respectively the degree of cell spreading, was found responsible for the regulation in the lineage commitment by modulating RhoA activity. RhoA is regulated upstream by mechanical cues and consequently actin-myosin tension. The absence of spreading, and hence cytoskeletal tension, induces the cells to become predominantly adipocytes, while spreading and cytoskeleton organization caused osteogenesis.

Increasing the density of cells in culture increases cell-cell contacts and decreases cell spreading, leading to growth arrest. As already pointed out, this fact was the early motivation for growing cells on adhesive islands capable of constraining the cell spreading. However, until now the studies were mostly focusing on the cell shape rather than on the effect of cell-cell contacts. Nelson and Chen recently provided new insights of this poorly understood phenomenon. By using specially designed patterns they were able to independently control cell-cell contact length and cell spreading. Surprisingly, they found that for the two types of epithelial cells tested, introducing cell-cell contact positively regu-
lated proliferation compared to single cells. Cell-cell contact increased proliferation by as much as three-fold in round cells and by 20% in highly spread cells. When the cells were separate by 5 μm (no physical contact) they behaved like single cells. This experiment could thus rule out that soluble factors were not the main cause for the cell-cell mediated increases in proliferation but suggested that physical contact was required for cooperative proliferation. The authors conclude that "contact inhibition" of growth of cells cultured in monolayers may be less a result of cell-cell contact and more a consequence of the decreased spreading as cells become crowded.

Neuroscience research also benefits from the micropattern technology; in literature they are often described as a powerful tool for studying neuronal cell function with an increase experimental reproducibility compared to non-patterned surfaces. Optimized geometrical patterns allows to induce directed cell differentiation as presented by Offenhäusser and coworkers. Also neurite growth on narrow patterned lines of adhesive chemistry allowed the formation of chemical synapses between neighboring cells. However, they reported that neurons grown on patterned substrates had a membrane capacity smaller than that of neurons on homogeneously coated controls, which they attributed to geometric restrictions. However, no differences were reported in terms of resting membrane potential or in the quality of synapses formed. Such bioengineered substrates are of potential significance in promoting peripheral nerve regeneration.

These selected example demonstrate that chemical micropatterns are unique tools to control and better understand the mechanisms leading to a specific cell phenotype and function.
1.4 **SURFACE CHEMISTRIES THAT RESIST THE ADSORPTION OF PROTEINS**

The ability to suppress non-specific interactions between the surface and the media is crucial in order to generate unbiased experimental outcomes. Also, preventing protein adsorption and cell attachment have emerged as a highly promising biomaterial modification for minimizing host-implant inflammatory responses. Advances in surface chemistry have made possible the synthesis of so-called non-fouling surfaces that significantly reduce the adsorption of proteins. Various, more or less effective chemical approaches have been published.

Several natural molecules have the ability to reduce the adsorption of proteins at surfaces; for example carbohydrates such as agarose and mannitol as well as albumin. Due to their limited efficiency and stability, a number of synthetic materials have been developed. The most widely used system is poly(ethylene glycol) or PEG: -[-CH$_2$-CH$_2$-O-]$_n$- also known as poly(ethylene oxide) or PEO. The factors governing protein resistance of a PEG-graft co-polymer (PLL-g-PEG) were recently investigated in details by Stéphanie Pasche (LSST, ETHZ). Many PEG surface-immobilization strategies have been demonstrated, for example, the grafting of two PEG chains at each end of an amphiphilic polymer such as poly(propylene oxide), forming a triblock co-polymer PEG-PPO-PEG. The hydrophobic PPO block is used as adsorption driving force on hydrophobic surfaces. This polymer class is also known as poloxamers or Pluronics® and has been extensively studied.

The development of micro-contact printing on gold coatings made the oligo-EG and the PEG-modified alkanethiolate self-assembled monolayers (SAM) one of the most frequently studied system. Although these PEG-modified SAMs show substantial reduction in protein adsorption, they still adsorb significant amounts of serum proteins. Moreover, they tend to oxidize under ambient
conditions and are thus, likely to lose their properties within a few hours only.\textsuperscript{105} Bearinger \textit{et al.} proposed an attractive alternative for modification of gold surfaces.\textsuperscript{106} Similar to the pluronics\textsuperscript{®} system, a poly(propylene sulfide) (PPS) central block with PEG chains on each side was proposed. This approach is currently being further developed in our laboratory by Lydia Feller (LSST, ETHZ).

Other polymer architectures such gels and polymeric self-assembled monolayers have also been successfully utilized to link PEG-chains to surfaces. For example, Healy and coworkers developed a gel-like interpenetrating polymeric network (IPN) of poly(acrylamide) and poly(ethylene glycol) [P(AAm-co-EG)].\textsuperscript{107,108} Toner and colleagues synthesized a poly(ethylene glycol) diacrylate (PEG-DA) hydrogel.\textsuperscript{109} Among the PEGylated polymeric monolayers, the poly(L-lysine)-g-poly(ethylene glycol), in short PLL-g-PEG, has proven to be a particularly attractive system. This polymer was initially developed for increasing the biocompatibility of microcapsules but and was later applied to flat surfaces.\textsuperscript{110} This polymer is a central component in this thesis and is presented in more detailed informations in chapter 3. Dalsin \textit{et al.} used a biomimetic linker, namely dihydroxyphenylalanine (DOPA), a key component of mussel adhesive proteins shown to strongly bind to titanium oxide as well as other types of surfaces.\textsuperscript{111,112} Ma \textit{et al.} modified alkanethiolates by polymerizing oligo(ethylene glycol) methyl methacrylate monomers by surface-initiated atom transfer radical polymerization (SI-ATRP).\textsuperscript{113} This living polymerization technique provides control over chain length (coating thickness) and surface density of the growing polymeric "bottle" brushes.

Silanes are commonly used as a surface-linker for PEG\textsuperscript{114,115} however, they are nowadays less popular in view of their hygroscopic behavior and tendency to polymerize and form island-like domains leading to heterogeneous coatings.
Lipid bilayers constitute another class of cell/protein repellent adlayers. The lipid composition can easily be tailored with differently charged lipids. Peptides and proteins can be embedded and thus provide interesting possibilities for patterning as being currently investigated by Fernanda Rossetti (LSST, ETHZ) and reported by others. Surface-immobilized lipid bilayers suffer one major drawback: they cannot be dried and must therefore always be stored in aqueous solution.

Surface immobilized polyelectrolyte multilayers (layer-by-layer (LbL) technique) have also been reported to resist the adsorption of cells under specific pH conditions.

A novel generation of polymers, able to switch between interactive and non-interactive properties upon change of temperature are emerging. A so-called "smart polymers" has been developed in the group of Okano: a thermo-responsive polymer based on poly(N-isopropylacrylamide) (PIPAAm) backbone with n-butyl methacrylate (BMA) grafted side chains was shown to have a temperature-induced phase transition (hydrophobic-hydrophilic) allowing the removal of entire cell-sheets upon lowering the temperature by a few degrees. Electrochemistry has been used by Mrksich and coworkers to dynamically control the surface properties for cell studies. This class of smart polymers is believed to be highly promising for future applications as enabling surface techniques to achieve not only spatial control over the chemistry (patterns), but also with the ability to change "on demand" local chemical properties.

The choice of protein/cell resistant chemistry is often dictated by the substrate material (glass, gold, titanium, etc). Some chemistries are highly versatile and can be adsorbed on different surfaces (e.g., PLL-g-PEG), while others require specific substrates as for example in the case of the gold-thiol system. However,
each approach has its specific strengths and weaknesses. Complexity of the synthesis, long-term stability (discussed in chapter 5) and compatibility with a specific patterning technique are typical aspects to be considered. Also, covalently bound molecules have higher binding strength than physisorbed adlayers; however, we will show in this thesis that non-covalent immobilization is a strategy that offers many attractive ways to modify surfaces.

It is interesting to point out that in the literature it is often reported (more or less clearly) that the use of serum (generally 10%) is restricted or banned when cells are cultured on micropatterns produced with a number of chemistries. Some groups completely exclude serum from the media while others initially plate the cells in serum-free conditions and later add the adequate serum. These measures are necessary in order to prevent the cells to attach to the background, which otherwise would result in poor cell pattern quality. We note that such protocols have been particularly frequently used in publications reporting the use of EG₃ or EG₆-modified alkanethiols. This is not so surprising since it is known that such coatings are not highly resistant to the adsorption of proteins.¹⁰⁴ Also, when nearly defect-free arrays of single cells are reported, they have been usually achieved with non-adherent cells.¹²⁶

The reader should also be aware that the challenges in patterning cells can vary with cell type; some cells are producing large amount of ECM proteins and require highly resistant coatings, while others are less delicate (extreme case are the non-adherent cell types). Unfortunately, this issue has never been addressed systematically in literature but is based on personal experiences and discussions with scientists active in this field. Studies on neuronal cells have significantly benefited of chemical micropatterns; however, neurons are relatively easy to pattern since they often require a specific protein to attach to (e.g. laminin) and are unlikely to grow out of such geometrically defined protein patterns.⁸⁵,⁸⁷,¹²⁷
1.5 CHEMICAL MICROPATTERNING METHODS FOR CONSTRAINING CELLS AT SURFACES

This section provides a review of existing micropatterning techniques dedicated to pattern cells in an organized and controlled manner. Some additional details can be found in our invited review (to be submitted) on micropatterning techniques for cell-surface interaction studies. Note that we did not make a strict distinction between the different adhesive chemistries used for each technique (ECM or peptides). The aim of this review was rather to provide a chemical micropattern manufacturing guide to scientists looking for the appropriate technique and chemistry to address their specific questions. Several excellent reviews focusing on the patterning of proteins, DNA, sugar, peptides and cells have been published elsewhere.6,18,61,62,128-134

Today, the most popular technology to create chemical patterns for cell-patterning is "soft-lithography". "Soft lithography" is not covering one specific method but rather a group of techniques with the common feature that at some stage of the process an elastomeric ("soft") material is used to create the chemical structures. In the family of "soft-lithography" two techniques are widely used, namely microcontact printing (µCP) and microfluidic patterning (µFP).

1.5.1 Microcontact printing

Microcontact printing (µCP) was originally developed for producing patterns for microelectronic applications.135 However, it’s potential was rapidly recognized for protein and cell patterning.77 Since then, there have been numerous publications about the usage of µCP for biological purposes and many useful methodological developments/variants were introduced. The popularity of the
method originates certainly from its simplicity, cheapness and flexibility. In brief, the process is performed as follows: First, an elastomeric stamp is formed by casting a liquid phase polymer over a topographical master. After curing/hardening, the stamp is removed from the master for "inking" with the molecules of interest. The stamping procedure upon which the "ink" is transferred to the substrate of choice is the most critical in terms of homogeneity and reproducibility. The final step (usually) consists of backfilling the non-stamped areas with a second molecule rendering the surface resistant to protein adsorption and cell attachment.

The starting point is a topographically structured master. This master is traditionally created by photolithography (Section 1.5.2) with micrometer-sized structures. The correct topological design of the master is a critical points of the technique (aspect ratio and spacings of the structures) and is mainly dictated by the mechanical properties of the stamp material. The most widely used stamp material is poly-(dimethyl)siloxane (PDMS) (mostly Sylgard 184 from Dow Corning) however, other siloxanes have also successfully been used for stamping. Recently, other materials like poly-olefin-plastomers (POP) or agarose gels have also been successfully applied. In most of the applications the PDMS stamps are "inked" after an oxygen/air plasma treatment to facilitate the adsorption of the printing molecules at the stamp surface. Two printing approaches are generally followed; the direct printing, where the cell adhesion molecules are directly transferred onto the substrate, and the indirect printing, where the functionalization is performed in two steps; first "non-bioactive" molecules are locally transferred and secondly followed by a functionalization step with the desired cell-adhesive molecules.
Indirect printing

Cells have been successfully patterned on gold (and silver) coated samples through the use of thiol based self-assembled monolayers (SAM), which covalently bind to the surfaces. This assembly system is by far the most widely studied and used; hydrophobic alkanethiolates are printed on gold substrates thereby creating hydrophobic patterns. In the second step the non-stamped areas are passivated (rendered protein/cell resistant) through the adsorption of oligo(ethylene glycol) (oligo-EG) coupled thiols. Upon incubation in a fibronectin solution (or any other cell-adhesive biomolecules), the proteins non-specifically adsorb on the hydrophobic regions. In order to retain the activity of the patterned molecules to a reasonable level Oliva et al. have chosen another approach; They patterned protein A with conventional μCP on glass substrates. Protein A is known to bind selectively to the Fc fragment of immunoglobulins. The patterns were thus, functionalized with a chimeric protein construct consisting of the extracellular domain of the axonal guidance molecule L1 recombinantly linked to the Fc fragment of IgG. Upon backfilling the surface with poly-L-lysine and plating neurons on the surface they observed a selective axon growth on the patterns, while the dendrites were found to grow on the background.

Direct printing

There is a wide range of approaches to directly pattern the substrates with cell-adhesion promoting molecules. In most cases different extra-cellular matrix (ECM) proteins or synthetic peptide constructs with ECM binding sites have been printed. The extensive literature reporting the printing of proteins and peptides indicate that the stamped molecules retain sufficient functionality to sustain cell attachment and spreading. The choice of ECM molecules to be printed is, to
a certain extend, dictated by the cellular system used for a particular experiment. For example, neurons were successfully patterned by using laminin or synthetic polypeptides containing the cell binding sequences of laminin\textsuperscript{85,142,143} or polylysine conjugated laminin.\textsuperscript{144,145} Patterning (and detection) of bacteria (E. coli) was achieved through the stamping of specific antibodies.\textsuperscript{146}

In the majority of the published studies the printing was used to define the cell adhesive regions on the first step, followed by "backfill-passivation" of the non-stamped areas. In some cases scientists have reported the stamping of the cell-repellent region first; Kam et al. stamped cell repellent octadecyltricholorosilane (OTS) onto silicon wafers to produce cell-free areas, while a subsequent adsorption of N1[3-(trimethoxysilyl)propyl]diethylenetriamine (DETA) was used to form the cell adhesive patches.\textsuperscript{144-146}

One of the strength of the \textsuperscript{1}μCP is its flexibility to pattern different substrates. By using the adequate surface chemistry, one can pattern/stamp surfaces such as gold, silver, various metals or metal oxides, glass, and different polymeric substrates. The quality of the \textsuperscript{1}μCP process highly depends on the mechanical properties of the stamp material as well as on the technical skills/experience of the scientist. The stamp must be soft enough to enable conformal contact with the substrate but sufficiently stiff to precisely reproduce the pattern at the surface and hence, avoid sagging and pattern deformation. These two competing parameters not only limit the resolution of the technique (smallest possible structure size and smallest separation between the structures) but also the maximum separation between the features (largest possible distance between two structures without sagging).\textsuperscript{136,147} In biological applications the smallest reported structure size is approximately 100 nm.\textsuperscript{138,148} However, such high resolution patterns are only achieved when using specific stamp material or by mechanically supporting the
stamp. As practice has showed, the smallest reproducible structure size with PDMS is \( \sim 1 \mu m \). A further limitation of the method is that under routine laboratory circumstances it is difficult to reproducibly and homogeneously pattern areas larger than \( \sim 2 \times 2 \text{ cm}^2 \).

1.5.2 Micro-fluidic patterning

Microfluidic systems are composed of channels, with typical width of 10-100 \( \mu m \), and sealed on a substrate. Microfluidic channels have largely benefited from the development of the PDMS casting technology and resulted in an extensive use of PDMS for producing microfluidic devices. The small channel width induce laminar fluid flow (Re \( \ll 2000 \)) within the channels and thus prevent the turbulent mixing of multiple parallel flows.\(^{131}\) These microchannels have been shown to be powerful tools for generating gradients of molecules in solution\(^{22}\) or at surfaces.\(^{56}\) Joyce Wong and coworkers recently reported that they have been able to produce a stiffness gradient on a surface. They observed that the cells preferentially migrated towards the stiffer region.\(^{56}\) However, due to the continuous nature of the channels such devices are limited in terms of pattern geometry. For instance, it is not possible to produce isolated patterns within a cell-resistant background. The geometry is limited to continuous lines (straight or curved) of cell-adhesive chemistry. Such geometry has proven to be highly interesting for the directed neurite growth. For example, Noo Li Jeon and colleagues build a microfabricated neuronal culture device with two chambers separated by micro-channels. Neuronal cells placed at one end of the channels were observed to grow straight neurites through the microtunnels and into the chamber situated at the opposite end.\(^{149}\) Such devices were shown to offer significant advantages over standard neuron culture methods since neurite growth can be guided with relative ease. For further details we refer the reader to two reviews on microfluidics.\(^{2,131}\)
In contrast to the previously presented soft-lithography techniques (microcontact printing and microfluidics), the focus of the following section is on photolithography-based approaches as well as some emerging techniques enabling the manipulation of single cells and potentially the assembly of individual cells for tissue formation in vitro.

1.5.3 Patterning with photolithography

In the photolithography process, geometric features drawn on a mask are transferred via UV illumination onto a substrate (see Microlithography Manual from Cornell Nanofabrication Facility\textsuperscript{150}). A mask is generally made of a quartz (glass) plate coated with a thin layer of non-transparent chromium containing the desired geometric features. The design of the mask is conveniently created with any computer-aided design (CAD) software and sent to a mask manufacturing company. Such quartz/chromium masks routinely allow feature resolution down to 1-2 micrometers. When poorer resolution is acceptable, e.g., with a tolerance of 10 \( \mu \text{m} \), one can easily produce a mask by printing the drawn features on a transparent foil by using a commercial inkjet printer. The foil is then taped onto a glass plate serving as the mask.

Photolithography has originally been developed for the fabrication of semiconductor devices. Therefore silicon wafers are widely used as substrate material. Besides of its finely tunable semiconductor properties, silicon wafers are atomically flat and thus convenient substrates for spin coating processes. For biological applications one has the possibility to use transparent glass wafers (quartz, Pyrex, etc) that have the advantage of being optically transparent and thus better suited for optical microscopy. Prior of transferring the patterns from the mask onto the substrate one needs to spin coat the wafer with a thin layer of
UV-sensitive polymer named photoresist (PR). A thin homogeneous layer of PR (usually in the micrometer range) is generated on the wafer surface by spin-coating. This is an efficient process for coating large surfaces with homogeneous thicknesses. Upon spin coating the PR coated wafer is placed on a hot plate for baking i.e., hardening the PR by solvent evaporation. The photoresist-coated wafer is then brought in close contact with the mask (in certain cases a gap between the two surfaces is preferred) in a mask-aligner device. The latter is equipped with a UV source and is activated when the wafer is aligned with the mask. A positive-tone PR is altered by UV light in a way that the irradiated regions become soluble and can thus be removed in the subsequent development process. Negative-tone photoresist become insoluble when irradiated and thus generates a negative image with respect to the mask. Upon development the surface is composed of patterns with "windows" providing access to the substrate (wafer) and a background protected with photoresist. At this point, any further processing steps will mainly be dictated by the type of chemical pattern one needs. Figure 1.2 illustrates the photolithography principles and two basic ways to produce chemical micropatterns.
Figure 1.2 1. A wafer, spin-coated with photoresist and illuminated through a mask. 2. In the case of a positive-tone photoresist the illuminated areas are dissolved by development and provide local access to the underlying substrate. One of two routes is generally followed from that stage: 3a. Evaporation of a thin layer of either metal or bioactive molecules (peptides, proteins, polymers, etc) and subsequent 4a. Lift-off in an organic solvent. Or 3b. Utilizing the patterned photoresist layer as a mask in order to locally etch through the metal (oxide) layer (that was deposited prior to photoresist spin-coating) down into the underlying substrate and subsequently 4b. Lift-off the residual photoresist.

For example Scotchford and coworkers created micropatterns simply composed of an inorganic contrast between two metals (covered by their respective native oxide layers) achieved by vapor depositing (CVD process) of the desired metal 1 prior to spin-coating photoresist onto the wafer. Subsequent to photoresist patterning, the wafer was again coated, this time with metal 2. As a final step the photoresist was simply removed by dissolution in an organic solvent with help of ultrasounds. This corresponds to route a. of Figure 1.2, which is also referred to the "lift-off" process. The patterned surface is finally composed of two metals (metal 1 = background; metal 2 = pattern). Interestingly, cells were able to sense differences between different metal oxides, particularly when aluminum in the background was combined with niobium, titanium or vanadium as patches: Shortly after cell plating the cells migrated and after 24h the aluminum surface was significantly depleted with cells, while the more cytophilic patches exhibited an increase in cell density. The preference of the cells for the metal oxide surfaces composed of TiO₂, Nb₂O₅ and V₂O₅ was correlated with the observation of significantly increased surface concentration of fibronectin (and correspondingly decreased albumin concentration) on those oxides in comparison to Al₂O₃. Such surfaces are relatively simple to produce but the pattern recognition by the cells is not perfect since the aluminum oxide surface is not intrinsically cytophobic and does not inhibit the adsorption of proteins. However, it is a clear demonstration that cells react sensitively to local variations in compo-
position and surface density of proteins, migrating to the areas with the higher concentration of fibronectin. Interestingly, cell attachment and spreading was not significantly different for the four metal oxides, when cells were cultured on the corresponding homogeneous metal-oxide samples.

Healy and coworkers \textsuperscript{152} produced line patterns of aminosilane/alkylsilane via photolithography and photoresist lift-off technique as presented earlier by Kleinfeld et al. \textsuperscript{127} The patterned photoresist were coated with a hydrophobic alkylsilane, followed by PR lift-off (Figure 1.2, route a.). The previously PR-protected background was subsequently coated with a hydrophilic (and charged) aminosilane. In the presence of serum the cells (rat calvaria osteoblast-like cells) showed a clear preference (higher cell number) for the aminosilane surface (charge and hydrophilic) compared to the alkylsilane. Interestingly, the bone-derived cells would only recognize the patterns if plated in serum-containing media or if the pattern was previously incubated with serum proteins and then exposed to cells in serum-free media. It is again very likely that the different affinity of the two silane-terminated surfaces for (specific) serum proteins is the driving force for the preferential cell attachment and organization on the aminosilane surface. Mineralization after 20 days of cell culture was almost exclusively restricted to the aminosilane line patterns. It is interesting to note that when the same experiment was performed with human osteosarcoma cells, no serum (or protein incubation) was needed for the cells to preferentially organize on the aminosilane surface. Bhatia et al. used a similar approach but directly immobilized the proteins in the "open windows" prior to photoresist removal. \textsuperscript{153} The major drawback with this approach (Figure 1.2, route a) is that the immobilized proteins are exposed to the organic solvent (acetone is usually used to dissolve the PR) and is known to denature proteins to a certain degree (depending on the protein). On the other hand, it was reported that the overall bioactivity of the immobilized col-
lagen I, after acetone PR lift-off, remained sufficient for supporting hepatocytes
growth. Since the aim was the production of a two-dimensional co-culture of two
different cell types (hepatocytes and fibroblasts) no passivating chemistry was
used after lift-off. This required the initial culturing to be performed under
serum-free conditions in order to be able to subsequently wash away the cells not
directly attached to the proteins. The second cell type was then plated and
allowed to attach to the glass substrate.

In an effort to create patterns with greater cytophilicity/cytophobicity contrast,
the combination of metal oxides pre-patterns (for example patterns of two differ¬
ent metals or oxides; route b. Figure 1.2) and advanced surface chemistry has
proven to be an efficient approach. The underlying idea is to exploit the specific
surface affinity of two classes of molecules and thus selectively adsorb molecule
A on oxide 1 (or metal 1) and the molecule B on oxide 2. Veiseh et al. specifi¬
cally immobilized thiolated self-assembled monolayer on gold patches and fur¬
ther functionalized them with NHS and EDAC chemistry for covalent attachment
of fibronectin. The SiO₂ background was modified with PEG-silanes in order to
reduce protein adsorption and cell adhesion. After 24 hours of culture and
upon rinsing, the 20x20 μm² squares of fibronectin were typically occupied by 4-
5 macrophage cells and no cells attached to the PEG background. Using gold sur-
faces as pre-patterns allowed the use of thiolated molecules, a class of molecules
extensively studied since the 1980’s (see Section 1.4) in the context of producing
self-assembled monolayers and microcontact printing applications (see
Section 1.5.1).

In order to circumvent the limitations inherent to alkanethiolates on gold
(Section 1.4), Michel et al. proposed a similar approach but based on pre-patterns
of two different, transparent metal oxides as illustrated in route b. of
Figure 1.2. In this technique, named Selective Molecular Assembly Patterning
(SMAP), the wafer is initially coated with two thin layers of oxides (titanium oxide and silicon oxide) prior to photoresist spin-coating. Upon PR development the wafer is placed into a reactive ion-etching device in order to locally etch the silicon oxide layer (outer coating) down to the underlying titania layer. After PR removal the resulting wafer is composed of patches of TiO$_2$ with a SiO$_2$ background and a step height of typically 10-20 nm. Two types of molecules are used to locally modify the surface, both based on simple dip & rinse processes in aqueous solution. First, the sample was dipped into a solution of ammonium dodecyl phosphate (DDP) which spontaneously forms a dense and order self-assembled monolayer on the TiO$_2$ islands. This selective adsorption exclusively occurs on the TiO$_2$ since the phosphate group has no affinity to the silicon oxide surface. The SiO$_2$ background is then rendered non-fouling by dipping the sample into a solution of poly(L-lysine)-g-poly(ethylene glycol) (in short PLL-g-PEG) for 30 min. This polymer spontaneously adsorbs from aqueous solutions onto negatively charged surfaces such as oxides of silicon, niobium, titanium and tantalum as well as tissue culture polystyrene. This polymer has shown to efficiently reduce the protein adsorption from serum to values $< 2$ ng/cm$^2$. Therefore, it is also non-adhesive for cells, whereas on the hydrophobic DDP-modified TiO$_2$ (-CH$_3$ terminated SAM) proteins adsorb strongly via hydrophobic exclusion. Consequently, the cells only attached and spread on the TiO$_2$, modified patterns. The protein layer adsorbed on the TiO$_2$-DDP originating from the serum forms a heterogeneous and partially denatured layer of proteins. Such chemically patterned surfaces benefit of the good spatial resolution achieved by photolithography as shown by the 1-micrometer-wide structures presented by Lussi et al.

The main disadvantage of the previously presented chemical patterning techniques is the lack of control on the composition and conformational activity of the protein layer as discussed in Section 1.2. Even in the case of a protein pre-
coating (e.g., fibronectin), dynamic events such as cell-surface remodeling by enzymes, cell-induced mechanical tensions as well as synthesized ECM proteins will alter the interfacial protein layer.\textsuperscript{161-164} Protein exchange between surface and solution (Vroman effect) will further contribute to modifying the initially immobilized protein layer.\textsuperscript{165,166} These spontaneous events can be limited or inhibited by covalently immobilizing the proteins on the surface as proposed by Veiseh \textit{et al.}\textsuperscript{154}

In a number of applications the activity of the cell-adhesive chemistry is secondary and simple cell-adhesive and non-adhesive patterns are required. For such needs researchers have investigated the possibility to immobilize the active biomolecules prior to photoresist spin-coating. This strategy is hampered by the incompatibility of the biomolecules with the photoresist developer (generally an alkaline solution). Bates and others elegantly proposed to protect the proteins with a thin layer of sucrose prior to PR spin-coating. This avoids the direct contact of the developer with the bioactive molecules during PR development.\textsuperscript{167,168} The major problem experienced with such concepts is the wafer processing temperature. The photoresist needs to be baked (often between 90 and 120°C) for hardening. Such temperatures are likely to compromise the integrity of the proteins, on the other hand insufficient PR baking will result in poor pattern resolution. As an alternative approach, a number of groups have recently proposed the synthesis of novel biocompatible photoresist formulations that do not require high hardening temperature and that are compatible with mild developing solutions.\textsuperscript{169,170} In brief, the biocompatible photoresist is locally altered by UV illumination through a mask and developed. Secondly, the whole surface (substrate and the photoresist) is coated with the desired biomolecules. Finally the photoresist remaining in the background is rendered soluble by a second short UV illumination step applied across the whole surface. Again, a mild aqueous
solution is used to remove the resist while preserving the already immobilized bioactive molecules. A major conflict in this approach is the need for a second UV irradiation step and the authors have not specifically addressed the influence of UV light on the immobilized molecules. It is known that UV light alters (crosslinking or oxidation) in a dose-dependent manner organic moieties and may thus affect their activity. However, small molecules such as biotin are less likely to lose their integrity and specificity upon short UV illumination in contrast to more complex and delicate biomolecules. Therefore, this process is probably better suited for small and stable biomolecules rather than for more delicate entities. Despite this limitation, such innovative photosensitive polymers have a great potential because they can be used to coat, develop and lift-off resists on a larger palette of substrates. For example, these resists can be lifted-off in solutions that are compatible with polymeric surfaces such as polystyrene for tissue culture (TCPS) or PET.

Ilic and Craighead proposed a way to get around the problem of the second UV illumination; instead of spin-coating the photoresist directly on the wafer they first spin coated a thin layer of parylene on the surface and next added a layer of PR. The overlying PR was then patterned and developed generating patches of parylene in a PR background. Oxygen reactive ion etching was subsequently used to remove the parylene layer not protected by the PR. The PR was then lifted-off in an organic solvent. Finally the surface is composed of oxide patches (substrate) surrounded by parylene. The surface can finally be coated with the desired protein solution and dried. Ilic elegantly proposed to remove the parylene template by mechanical peeling. Besides of avoiding any contact between the biomolecules and the organic solvent, the dry lift-off allowed to pattern cells on 2 μm wide lines with high fidelity. Orth et al. applied this technique for patterning cells on lipid bilayers down to 1.5 μm pattern size.
Creating regular arrays of single cells (excluding cell-cell contacts) have become increasingly attractive. For example, it is known that physical cell-cell contacts upregulate proliferation in endothelial and smooth muscle cells. Therefore, if one aims at studying the specific influence of substrate chemistry (by excluding cell-cell contacts) on cell survival, growth and differentiation, it is necessary to use arrays of isolated cells. If individual cells are to be studied by optical methods, measuring for example size and shape of focal adhesions, perfect cell arrays are not required, meaning that selected islands containing single cells can be visually selected. Such approaches have proven in the past to provide important insights in specific cell mechanisms (see Section 1.3). With the ongoing development of techniques allowing the study of cells at the individual level (in situ PCR, molecular beacons, etc), such approaches are believed to be very promising. Producing large arrays (hundreds or thousands) of single cells confined to identical geometrical islands will then become essential, ensuring an "identical" microenvironment for each cell of the population. Generating defect-free large arrays of single cells remains a challenging task (see Section 5.2). The emerging field of cell-based sensors would greatly benefit from such capabilities because the ability to address individual cells in an automated way would enable the recording of cell responses upon stimuli (electrical or pharmacological). Decreasing the size of the adhesive spot in order to physically accommodate only one cell is one approach towards this goal (see Section 5.2). Dielectrophoresis was recently presented as an interesting strategy for generating arrays of one or two cells per island with relatively large adhesive areas (>100 μm²). Gray et al. fabricated a photolithography based device able to generate local electric forces for capturing cells on specific regions of the surface. Although the approach proposed requires some know-how in microfabrication, each processing step can easily be carried out in any conventional clean room.
The idea is to generate one or two local electrical field sources centered below each adhesive island and actively attract the cells to the patterned surface. The cells in the electric field are polarized and directed towards the electrical field maxima, located at the site of the adhesive pattern. The cells then attach and spread on the fibronectin islands that are surrounded by cell resistant Pluronics® molecules. One interesting aspect of dielectrophoretic cell patterning is the active guidance of cells towards the patterns rather than relying on the random cell seeding and cell migration onto the adhesive areas. Other techniques based on the active placement of individual cells are discussed in Section 1.5.7 and Section 1.5.8 on "jet patterning" and respectively “Laser guided patterning and cell printing”.

### 1.5.4 Plasma polymerization combined with photolithography or laser ablation

Plasma polymer technology combined with photolithography has been exploited over the past years for the patterning of biomaterials dedicated to 2-D in vitro cell cultures. A plasma is an ionized gas where some or all the electrons of the outer atomic orbitals have become separated from the atoms or the molecules. Artificial plasmas are often generated by radio-frequency (MHz) glow discharge setups. Provided that plasma parameters are adequate, they allow deposition of polymeric films with thicknesses ranging from several nanometers up to micrometers. In the work by Hoffman and coworkers it is demonstrated that plasma lithography can be a valuable alternative to other techniques. Polyethylene terephthalate (PET) substrates were first coated with a non-fouling plasma polymer of tetrabutyrate (tetraethylene glycol dimethyl ether). Standard photolithography procedure was then applied as described in the previous paragraph (photoresist spin coating, UV illumination through a mask and develop-


ment of the structures). The resulting surface composed of tetraglyme polymer patches surrounded by PR is then coated by a fluorocarbon (FC) plasma-deposited polymer. Subsequently, the photoresist is lifted-off and the final surface is composed of hydrophobic cell-adhesive patches (FC) in a non-interacting hydrophilic background. Smooth muscle cells (SMC) were seeded on these surfaces initially without serum in order to avoid cell spreading on the tetraglyme background. For longer time culture of SMC on patterns (10x100 μm²; 40x40 μm²; 20x20 μm²), 10% CS (calf serum) was added to the media 24 hours after seeding. Cells were shown to maintain pattern fidelity at least for 14 days.

A challenging aspect of plasma polymerization is the mechanical stability of the deposited layer. Delamination risks can be reduced by increasing the cross-linking degree between the polymer (tetraglyme) and the substrate (PET). Roughly, the higher the plasma energy is, the more the polymer will be crosslinked to the surface. The drawback of a high-energy plasma is a greater loss of functionality (in this case resistance to protein adsorption). This is especially true when a final lift-off step has to be performed in an ultra-sonicated bath. To allow more freedom for the processing parameters, the first plasma polymer can be deposited in two steps, first a high energy plasma layer that displays good adhesion properties to the substrate, and secondly a lower energy plasma deposition for good chemical functionality.

An alternative approach to the photolithographically based patterning of plasma polymers was proposed by Thiessen et al. Here a cell-resistant PEO based plasma polymerized surface was locally ablated via laser and thus rendered protein/cell adhesive. Collagen I was pre-adsorbed on the ablated patterns and bovine corneal epithelial cells were shown to attach and spread exclusively on the protein patterns. This laser ablation technique allows creating sub-cellular-sized features down to 1 μm size. Yamato et al. applied laser ablation on elec-
tron-beam grafted cell-repellent polymers (PIPAAm). It was reported that hepatocytes could be cultured on 50x50 μm² patterns of Fn pre-coated TCPS for at least two weeks.

Plasma lithography is a very versatile and useful technique in the sense that a great variety of substrates can be used to create plasma polymer films; moreover, plasma polymerization is compatible with large-area surface treatment, provided the equipment used has been designed accordingly. The use of photolithography (photoresist and UV) makes the process efficient and reproducible over large scale. As for every technique there are specific limitations; it is usually difficult to produce highly stable plasma polymer layers. Finding the optimal conditions to prevent delamination is considered to be relatively time consuming compared to a simple dip&rinse processes. We believe that plasma lithography is not (yet) a standard technique to produce reliably surfaces with well defined surface chemistries in terms of biofunctional groups.

1.5.5 Photoimobilization (photochemically generated patterns)

Surface-immobilized photoreactive molecules have been used for generating chemical micropatterns for cell attachment. Hu and coworkers immobilized oligopeptides containing the Arg-Gly-Asp (RGD) sequence on top of ethylene glycol modified-alkanethiols via UV or laser activation of benzophenone groups. They showed that a linear correlation exists between the exposure time and the amount of immobilized oligopeptide (in the range of 0 to 10 pmol/cm²). By scanning the laser at different velocities (different illumination times) they successfully created peptide gradients inside the micropatterns. After 24 hours in serum-free media more fibroblasts attached to the high peptide density patches.
compared to the more sparsely immobilized peptide patterns. This technique is highly interesting since it allows to control the RGD surface densities within each adhesive island. However, the chemistry applied restricts to culture cells in serum-free media. More recently Dillmore et al. used a similar approach where a hydroquinone group (linked to tri(ethylene glycol)-alkanethiol SAM) was rendered accessible by photochemical deprotection of its NVOC entity.\textsuperscript{181} Subsequently the hydroquinone group was reversibly oxidized to benzoquinone by applying an electric potential. Finally, peptides were covalently immobilized to the activated areas via Diels-Alder reaction thus providing cell-binding sites. 3T3’s Swiss fibroblasts were seeded in serum-free media for 4 hours before the media was exchanged for serum-containing media. The cells were shown (24 hours after cell seeding) to follow the RGD peptide patterns. It was reported that the cells bound specifically to the peptides as demonstrated by cell detachment upon addition of soluble, competitive RGD peptides.

Three different methods were used to locally photoactivate the surface. In\textsuperscript{180} a photolithography mask was used, potentially allowing downsizing to 1 μm features. Laser light photoactivation was also reported by using a beam of 20 μm in diameter. The advantage of the laser-beam activation is the ability to create heterogeneous patterns by varying the exposure time, as well as complex 3D structures as shown by Shea and coworkers.\textsuperscript{182} However, this process is much slower in comparison to the massively parallel production capabilities of photolithography. In\textsuperscript{181} microfiche masks were used to demonstrate the feasibility of the parallel processing method. The second strategy was a scanning type of lithography where the surface was directly activated through a microscope objective and a mercury lamp. The authors conclude that such serial photoactivation could produce high-resolution patterns when activated with techniques such as scanning near-field optical microscopy (SNOM).
The above-presented techniques have the merit to be highly flexible with respect to type and density of the immobilized ligands. However, they are probably not well suited for long term (>24 hours) cell culture since the thiols are likely to oxidize under ambient conditions.\textsuperscript{105,106}

1.5.6 Stencil-assisted patterning

A stencil is a membrane (stiff or flexible) that is structured with through-holes of the desired size and geometry.\textsuperscript{131,183-186} When the stencil is brought in close contact with the substrate it can be used as a template to locally modify the surface while the areas outside the holes remain protected by the stencil. Processes such as deposition or ablation of molecules for chemical patterning as well as cell seeding (without any chemical surface treatment) will therefore only occur on the areas beneath the holes. Stencil materials range from soft membranes (e.g. PDMS) to highly stiff grids (e.g. metal-based). For example, Folch \textit{et al.}\textsuperscript{187} used elastomeric stencils (PDMS membranes produced by photolithography) to directly pattern cells without any chemical modification of the surface. This simple process offers the advantage of not requiring specialized tools or chemistry once the master template is available. The stencil is sealed onto the surface prior to cell seeding. Upon cell attachment the stencil can be manually peeled-off. With this approach each local cell population can rapidly grow out of the pre-defined areas if no cell-resistant chemistry is present between the patterns. Therefore model surfaces prepared in this manner are potentially interesting to study the migratory behavior of cells as well as creating co-cultures of two (or more) cell types where the first cell type attaches and spreads in the holes delimited by the stencil, while the second type spreads on the background once the stencil has been removed. Tourovskaia and coworkers\textsuperscript{188} exploited the convenience of the
PDMS stencil in conjunction with a non-fouling substrate. A homogeneous layer of grafted interpenetrating network (IPN) of poly(acrylamide-co-ethylene glycol) (PAAm-co-EG)\textsuperscript{189} was polymerized and cross-linked on a glass surface. The stencil applied onto the polymer layer enabled the local etching (O\textsubscript{2} plasma) and hence, removal of the protein/cell resistant coating in islands defined by the stencil features. This highly swellable IPN resists the adsorption of proteins and confers stability to the cell patterns up to 60 days\textsuperscript{189}.

More recently Brugger and coworkers proposed a procedure to produce soft, low residual stress shadow-masks (stencils) with modified SU-8 photoresist\textsuperscript{190}. The stencils are composed of a photolithographically patterned thin membrane (5 \textmu m) and a frame membrane of 150 \textmu m for mechanical stability. They created patterns composed of different deposited metals through multistep evaporation and stencil realignment. After the first evaporation the shadow-mask is displaced and realigned with a precision of 2 \textmu m with the help of jigs and alignment pins before the next evaporation is performed with another metal. This process can virtually be carried-out indefinitely and is thus very interesting for patterning different materials on the same substrate with a high spatial resolution. This concept was also applied for the production of a new generation of stencils that combines micro and nano features (100 nm) produced by focused ion beam milling\textsuperscript{191}. We believe that such stencils have a great potential for biological applications, such as protein/peptide immobilization and cell patterning. Although it requires some technical skills to align the flexible stencil on the surface (with respect to electrodes or underlying chemical patterns) and ensure an adequate sealing the technique is a simple, large scale and cost-effective replication technique. In addition, the stencil technique offers two significant advantages because it is suitable to virtually any substrate material (metals, oxides and stiff/soft polymers) as well as curved surfaces. Also, the absence of organic solvents makes it an interesting
1.5.7 Jet patterning

Ink-jet printing is another example where tissue-engineers have adapted existing technologies for biological research and applications. For example, it is possible by minor modification of a commercial printer to create chemical patterns by directly jetting alkanethiols or proteins onto surfaces.\textsuperscript{192-195} Wilson \textit{et al.} reported direct printing of cells onto gel membranes with a reported dead-cell percentage of 25\% after 72 hours. Cell death was primarily attributed to dehydration of the cell microenvironment (drying of the drop) rather than the passage through the needles.\textsuperscript{196} The ink-jet technology has the advantages of being inexpensive, flexible in terms of substrate choice and fast. Furthermore, multifunctional surfaces can be created by using a multiple nozzle device for printing different proteins for example. It is also sufficiently precise to print multiple compounds on the same spot. However the spot size and resolution is greatly limited by the printer resolution, the nozzle diameter and the liquid/solid interfacial tension. The smallest reported spot by ink-jet technique is approximately 100 μm. We believe that this technique is potentially interesting to create large arrays of single cells if combined with surfaces patterns composed of cell-adhesive and cell-repellent areas. The challenge is to precisely spot each cell on an adhesive island (or sufficiently close to it) in order to be able to generate, in a high-throughput manner, arrays of single cells.

1.5.8 Laser guided writing with cells

Optical force is another way to direct living cells to specific locations on surfaces. Objects such as cells or particles being constituted of matter with higher
refractive index than the environment and submitted to focused laser light will be confined into a so-called optical trap. Such “lock-ins” are not necessarily punctual as the laser tweezers method but can be straight lines, defined by the laser beam and the low numerical aperture lens. Odde and Renn have shown that it is possible to continuously propel cells along a light path of ~300 μm length and create clusters of cells on a target surface. The laser beam continuously captured cells as they drifted into the light path by natural convection in the fluid medium. The optical forces exerted on the cells originate from the interfaces between two different refractive indexes. Each time the light is redirected at the interface, the cell is redirected in response and the total momentum is conserved. Forces in the order of a few piconewtons were reported. The further away from the light source the more the beam will be divergent resulting in smaller optical forces. Above ~300 μm the natural convection force of the media dominates over the optical force, thus no longer allowing control over cell position. This limitation has been overcome by guiding the cells inside a hollow optical fiber allowing for total internal reflection of light. Control of cell placement across distances as large as 7 mm have been reported with traveling speeds of 50 μm/s when using optical fibers with an inner diameter of 100 μm. With the recent development of an advanced spatial light modulator, Glückstad and coworkers were able to micromanipulate in real-time multiple particles and living cells. Given an adequate set-up, they could arbitrarily move each optical trap with the “mouse-cursor” of the computer. The parallel and real-time working capabilities of such devices are believed to have a future potential for cell patterning and tissue engineering.

As for the ink-jet cell-printing technique, the laser-guided method has the advantage to potentially create patterns with different cell types and thus engineer heterogeneous tissues. Furthermore the laser-guided writing benefits from a
resolution (~1 μm) much better than for the ink-jet method (~100 μm) and thus allows a higher degree of spatial organization. This non-contact patterning method is potentially suited for building up successive layers of cells to create three-dimensional networks or tissue. However, one important aspect of this technique that remains poorly investigated is the question to what extent the cells become damaged by the laser light? Odde et al. reported that using near-infrared light the patterned cells were viable, but it is not clear whether functional biomolecules were altered during the process. It has also been reported that higher input power levels are required when large arrays of cells are to be manipulated (in parallel) because of the low differential index contrast to the surrounding medium and the irregularity of cell morphology. The need for relatively high laser power might induce irreversible alteration in the cells. Also, once the cells are in contact with the substrate, they will spread and migrate randomly and thus, rapidly transform the originally written pattern, unless specifically designed surface chemistry is combined with this cell-deposition technique.
CHAPTER 2

Scope of this thesis

This thesis project was motivated by the need for better defined, chemically patterned surfaces in the biosciences in general, and in particular for the study of fundamental mechanisms occurring between living cells and artificial surfaces. The patterning process invented and developed within the frame of this thesis, named Molecular Assembly Patterning by Lift-off (MAPL) has been inspired by previously established techniques, in particular Selective Molecular Assembly Patterning (SMAP) (Section 1.5.3), developed by Roger Michel and Jost Lussi (both LSST, ETHZ). Patterning of surfaces into interactive and non-interactive areas has proven to be a very useful technique to study the behavior of single cells or ensembles of few cells, thus reducing the complexity of the system in comparison to the very heterogeneous character of cells cultured on homogeneous substrates. To be successful, such cell-pattern approaches rely on the ability to host cells in well controlled microenvironments. In this context, there is still an important need to develop patterning techniques with improved definition of the biointerfacial properties. We have learned that the development of a particular cell phenotype is mainly dictated by surface cues such as type and density of ligands, substrate stiffness, topography/roughness and cell spreading. All these cues together steer the signaling processes occurring within the cells. Analyzing
data of such experiments is often of limited value since typically only one of these cues is controlled and investigated, while the others are "working in the dark", blurring our understanding of the complex interplay of the various factors affecting cell behavior at surfaces. Therefore, the need for improved quantitative methods that allow independent control over different surface parameters, is widely recognized in this research community.

The development of the Molecular Assembly Patterning by Lift-off (MAPL) technique was motivated by the endeavour to overcome limitations inherent to the existing patterning techniques. The specific requirements were:

- The cell-adhesive chemistry (bioligands) should elicit only specific interactions with the media.
- Quantitative control over type and surface density of the biointeractive ligands (with the possibility to immobilize multiple types of ligands).
- Pattern geometry tunable independently of the ligand chemistry, with a wide accessible range of pattern dimensions.
- Reproducible over large scale (tens of cm).
- Compatible with transparent substrates (for optical microscopy and sensing applications).
- Rapid and cost-effective (e.g., no reactive ion etching).

Overview of the thesis chapters: Chapter 1 provides an overview of the many different technological approaches described in the literature to produce patterns at the micron scale and their application to produce and study cell patterns. One of the main conclusion is that, despite the great progress made in the past 20 years in deciphering cell-biological mechanisms, there is still a considerable lack
of understanding, at the mechanistic/molecular scale, the factors that govern cell-surface interactions. **Chapter 3** presents the MAPL process based on the combination of top-down photolithography and bottom-up self-assembly of PLL-g-PEG and its functionalized derivatives. The characterization of each step of the MAPL surface modification is described in detail; the systematic optimization of each step resulted in the establishment of a robust protocol (**chapter 4**). Cell culture investigations on such patterned surfaces are discussed in terms of pattern stability and cell adhesion (**chapter 5**). The MAPL technique is not limited to cell patterns but can be utilized for numerous applications in the biosciences. To illustrate this we demonstrate the feasibility to produce patterns of DNA-tagged vesicles (**chapter 6**) and protein microarrays (**chapter 7**). **Chapter 8** focuses on the extension of the MAPL technique from the micron to the nanoscale. 100 nm chemical patterns were produced by a combination of nanoimprint lithography and chemical surface functionalization. **Chapter 9** provides conclusions and an outlook on the different aspects related to the development of the MAPL process and its applications. Experimental details and instrumental information are found in **chapter 10**.
CHAPTER 3

Molecular Assembly Patterning by Lift-off (MAPL)

This chapter presents the novel biochemical patterning technique developed in the frame of this thesis as well as the chemistry used to locally functionalize the surface. The latter is based on the grafted copolymer poly(L-lysine) grafted poly(ethylene glycol) (PLL-g-PEG). The two main characteristics of this polymer adsorbed on surfaces are its ability to resist the adsorption of proteins and its electrostatic interactions with the surface. PLL-g-PEG has been extensively used and studied within the Laboratory for Surface Science and Technology (LSST-ETHZ) over the past 5 years (over 35 publications, published or submitted).

The technique developed in this thesis was published for the first time in 2004 in Advanced Functional Materials.\textsuperscript{201}

3.1 Molecular-assembly via PLL-g-PEG

PLL-g-PEG is a copolymer composed of a polylysine polycationic backbone carrying amine groups that interact with negative charges on the substrate. The hydrophilic grafted PEG side chains extend at the water-polymer interface and are responsible for preventing non-specific protein adsorption. At neutral pH, the PLL backbone carries positive charges (the free amines that have no grafted
PEG) and are exploited for the electrostatic immobilization onto negatively charged surfaces (IP of PLL ~10). With the adequate amount of grafted PEG chains one can drastically reduce the adsorption of proteins from full serum to < 2 ng/cm².

For the PLL-g-PEG user this polymer has multiple advantages besides of its outstanding protein resistance and its straightforward synthesis. For example, the coating of negatively charged samples is done by simply dipping the surface in an aqueous solution of PLL-g-PEG for 30 minutes. Depending on the initial concentration of the solution, multiple surfaces can be coated with the same solution thus making it a cost-efficient coating process.

By controlling the synthesis parameters one can tailor the grafting ratio of the PEG chains on the PLL. The grafting ratio (g) is defined as the number of lysine monomers per PEG chain. A low grafting ratio will result in a high PEG density polymer while a high grafting ratio will produce a polymer with a more open PEG structure. The architecture of the copolymer can be further modified by changing the molecular weights (commercially available products) for both, the PLL (2, 20, 200 kDa, note that a polydispersity of 1.1-1.3 has been measured for PLL 20 kDa) and the PEG (1, 2, 5 kDa). A large library of polymer architectures has been produced and thoroughly characterized in other works.⁹⁰,⁹¹,¹¹⁰ These results were important for selecting the polymer architecture to be used in this thesis. The key criterion was the protein resistance and therefore the following architecture was used: PLL(20)-g[3.0-4.0]-PEG(2). All batches used in this study had a grafting ratio (determined by NMR) between 3 and 4 as shown in Table 3.1. Functionalized PLL-g-PEG variants were used in order to elicit specific interactions between the surface and the environment. A biotin-modified polymer was used as a model system to characterize the patterns. The high affinity binding between biotin and streptavidin was exploited in this case. For cell
patterns a peptide modified PLL-g-PEG was used. A linear 12 amino acid sequence containing the RGD (Arg-Gly-Asp) cell-binding domain was grafted onto the PEG-arms, as shown in Figure 3.1. It is possible during synthesis (with some constraints) to control the number of functionalized PEG chains on the polymer. The PEG chains coupled to bioactive groups have a larger molecular weight (3.4 kDa) than the non-functionalized PEG chains (2kDa). These longer chains should favor the ligand presentation to the surrounding media and reduce the possibility of having ligands embedded in the brush PEG structure.

Table 3.1  The polymers used in this thesis

<table>
<thead>
<tr>
<th>Architecture</th>
<th># of bioactive groups per polymer molecule</th>
<th>polymer Mw [kDa]</th>
<th>batch</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLL(20)-[3.3]-PEG(2)</td>
<td>-</td>
<td>67.7</td>
<td>SP09</td>
</tr>
<tr>
<td>PLL(20)-[4.7]-PEG(2)/PEG(3.4)-biotin (50%)</td>
<td>19.2</td>
<td>74.3</td>
<td>FD10</td>
</tr>
<tr>
<td>PLL(20)-[3.7]-PEG(2)/PEG(3.4)-RGD (6.5%)</td>
<td>1.6</td>
<td>65.2</td>
<td>MS007</td>
</tr>
</tbody>
</table>

The grafting ratios [g] have been determined by NMR. The percentages indicate the amount of PEG chains that are functionalized with either a biotin or a peptide group. All three types of polymers reduced adsorption of serum proteins to < 2 ng/cm². The lyophilized polymer (white powder) were dissolved in "Hepes 2" (4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid, adjusted to pH7.4 with a 6 M NaOH solution and complemented with 150 mM NaCl) and adsorbed on surfaces at 0.1 mg/ml for 40 minutes.

Details of the synthesis of the PLL-g-PEG⁹¹ and its functionalized derivatives have been carefully documented elsewhere.⁴⁸,₁⁶⁰ A short compilation of the polymer synthesis protocols can be found in chapter 10.
The molecular weights of the peptide and the biotin vitamin were, respectively, 1081 Da and 244 Da.

3.2 THE MAPL PROCESS

The micropatterning techniques are generally closely related to the specific type of chemistry one desires to apply to the surface. The challenge is to find a combination of substrate, patterning method and chemistry that are compatible and allow to create the desired patterns. Many material parameters and the specific goal of the chemical pattern together, define which approach is best suited; a
technique that works with non-transparent substrates, such as silicon or gold, will not necessarily be compatible with a transparent substrate and vice versa. The motivation behind the development of the MAPL technique was fed by a number of criteria that cannot be met by any other existing patterning technique. The process should have to: i) be compatible with transparent substrates; ii) allow quantitative control of the surface density of the bioactive groups within patterns of micrometer dimensions; iii) elicit purely specific interactions between the surface and the environment via molecular recognition out of a complex mixture of proteins (serum or plasma); iv) allow flexibility in terms of the type of ligand to be immobilized; v) be a cost- and time-effective process not requiring expensive instrumentation such as reactive ion etching (RIE) but still allow the use of batch processes for the reproducible production of a large number of samples in a reasonably short time; vi) allow the precise alignment of the patterns with respect to the chip edges; vii) be potentially applicable to sub-micrometer features for creating nanopatterns. The MAPL process has been inspired by a variety of earlier patterning methods published in literature over the past twenty years. What makes the MAPL technique innovative is the combination of standard photolithography steps with the use of PLL-g-PEG for creating biochemical patterns with unique features as listed above.

The MAPL processing steps are presented in Figure 3.2 in a summarized fashion. The complete technical protocols can be found in chapter 10. The initial processing part takes place preferentially in a clean room where a wafer (Pyrex or silicon, sputter coated with Nb₂O₅) is spin-coated with photoresist (PR) and patterned by UV illumination through a mask and subsequent development. At this stage, the wafer is composed of resist-protected and -unprotected areas (stage I). The wafers are then diced into 1x1 cm² samples and stored until use. The surface patterning is carried out in the lab where the samples are sonicated in a ultrapure
water bath for 5 minutes, blow-dried with nitrogen and cleaned with oxygen plasma for 10 s. Immediately after, the sample is incubated in a solution of PLL-g-PEG/PEG-X with a fraction of PEG side-chains functionalized with a bioligand X (biotin or a short peptide sequence) (stage II). Upon polymer adsorption, H$_2$O rinsing and drying, the sample surface is rinsed with a flow of N-methyl-2-pyrrolidone (NMP) that dissolves the PR and hence partially flushes away the functionalized PLL-g-PEG bound to the photoresist. The resist is completely removed by placing the sample into three successive beakers filled with NMP, all these steps are performed in a sonicated bath. First, the sample is dipped into a beaker (4 ml NMP), after 30 s, 1 ml is pipetted out and the same amount of fresh NMP is added. After 1 min the sample is transferred (rapidly) into a new beaker containing 4 ml of fresh NMP for 3 min. Subsequently the sample is transferred to a beaker containing 2 ml of NMP diluted in 2 ml of ultrapure water for 1 min. The surface is then transferred into an agitated bath of ultrapure water (3-5 min) and nitrogen blow-dried. The resulting surface is composed of islands of PLL-g-PEG/PEG-X in a niobia background (stage III). In a final backfill dip-and-rinse step, non-functionalized PLL-g-PEG is adsorbed on the oxide background rendering it resistant to non-specific protein adsorption (stage IV). At this stage the sample is ready for either incubation in a protein solution or a cell suspension. If desired, the sample can be quickly flushed under a stream of ethanol for sterility purposes.
Figure 3.2 Schematics of the MAPL processing steps. The image on the left side illustrates a wafer after photolithography in the clean room, i.e. the desired patterns are transferred from a mask into a photoresist via UV illumination and subsequent development. The right side of the figure shows the steps for surface patterning carried out in the lab.

Photolithography can be used to achieve 1 μm resolution features. Such subcellular sized patterns are interesting, e.g., for studying integrin clustering and shape of focal adhesion complexes. For nanopatterning via MAPL the reader is referred to chapter 8 or to our paper published in Nanoletters.
CHAPTER 4

Characterization and optimization of the MAPL process

4.1 HOMOGENEOUS ADLAYERS

Preparation and characterization of homogeneously coated surfaces (non-patterned) was used to optimize each of the two key steps of the MAPL protocol: i) the quality of the photoresist removal step and ii) the potential loss of PLL-g-PEG during the lift-off process. X-ray photoelectron spectroscopy (XPS), optical waveguide lightmode spectroscopy (OWLS) and ellipsometry (ELM) were crucial in providing guidance to the establishment of a robust protocol in terms of reproducibility and controlled surface chemistry. The results and information gained on these homogeneously coated surfaces were then extrapolated and used to create the micropatterned surfaces. The experimental and instrumental details can be found in chapter 10.

4.1.1 Quality of the photoresist lift-off

4.1.1.1 XPS RESULTS

XPS was used to assess the quality of the photoresist lift-off on niobium oxide coated wafers. The samples used in this study were homogeneously coated with Shipley S1818 photoresist, no UV illumination step was performed. Note that the
results presented in this section reflect the optimized lift-off process. Figure 4.1 shows two XPS survey spectra of the photoresist and a surface after lift-off in NMP. The spectra of the sample coated with PR did not show the underlying niobia since the PR thickness is \( \sim 1.7 \ \mu \text{m} \) and the information depth of XPS is approximately 10 nm.\(^{203}\) The major signals were attributed to carbon 1s and oxygen 1s peaks. Traces of Si and F were also detected. The exact composition of the PR is not publicly known; however, information on the major constituents is given in chapter 10.

The surface after lift-off did show very little hydrocarbon residues (9%) indicating that the PR was removed efficiently. As a control, a niobia-coated wafers was oxygen-plasma cleaned (2 min) and measured. Both types of samples were always rapidly inserted into the ultrahigh vacuum chamber of the XPS after processing (within 5 min) in order to avoid hydrocarbon contamination from the ambient environment. Figure 4.2 depicts the carbon 1s peaks of a plasma cleaned surface and a PR lifted-off surface. A difference in the peak shape is observed, the peak-shoulder at 286.5 eV, attributed to the C-O component, was more pronounced on the plasma cleaned surface.

*Figure 4.1* XPS survey spectra of the photoresist and the niobia surface after lift-off.
Characterization and optimization of the MAPL process

Figure 4.2 Detail spectra of carbon 1s signals of a niobia surface after oxygen plasma cleaning and after lift-off.

These differences are not significant since the elementary compositions only differ by 1-3%, suggesting that a complete PR removal has occurred during the lift-off process (Table 4.1).

Table 4.1 Atomic percentages measured by XPS on a plasma cleaned reference surface and a MAPL sample for which the photoresist was removed by NMP. n=4 for each sample type. Peak areas of C1s, O1s and Nb3d were used as a basis for quantification.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>% C</th>
<th>% O</th>
<th>% Nb</th>
</tr>
</thead>
<tbody>
<tr>
<td>O₂-plasma</td>
<td>7</td>
<td>64</td>
<td>29</td>
</tr>
<tr>
<td>lift-off in NMP</td>
<td>9</td>
<td>61</td>
<td>30</td>
</tr>
</tbody>
</table>

Lift-off experiments were also conducted in acetone instead of NMP. A number of criteria made us rapidly discard acetone as a photoresist remover: even though the transfer from one solvent beaker to the next can be executed rapidly, the low boiling point of acetone (56°C vs. 202°C for NMP) induces partial drying of the surface during the sample transfer. Such surface drying prevents proper
removal of the photoresist and results in an incomplete lift-off, leading ultimately, to a poor PLL-g-PEG adsorbed layer. Moreover, NMP in contrast to acetone, is reported to have a low toxicity, rapid biodegradation and 100% solubility in water.\textsuperscript{204}

The same lift-off protocol was also applied to S1818 photoresist coated on various oxides such as SiO\textsubscript{2}, TiO\textsubscript{2} and In\textsubscript{2}O\textsubscript{3}-SnO\textsubscript{2}, all showed similar hydrocarbon residual percentages to their plasma cleaned equivalents. Note that we have observed that the adhesion of the S1818 PR on silicon wafers with a natural SiO\textsubscript{2} layer (no sputter coated oxide) is very poor, leading to PR delamination during incubation in Hepes 2 buffer. This problem can be circumvented by spin-coating S1818-SP16 PR. The SP16 denotes a higher adhesion strength which is usually achieved by addition of silane molecules in the final PR composition.

4.1.1.2 Auto-fluorescence of S1818 photoresist

We have observed that the S1818 PR is auto-fluorescent when excited at a wavelength of 488 nm (also at 543 nm, but with lower fluorescence intensity). No auto fluorescence was observed at 688 nm. Fluorescence measurement can be an extremely sensitive detection method. Therefore, we tried to detect traces of PR upon lift-off with a confocal laser scanning microscope (CLSM) by applying high laser power as well as high photomultiplier (PMT) gain.

Figure 4.3 shows a sample with a PR pattern clearly visible by auto fluorescence when excited at 488 nm (longpass filter 505 nm) and the same sample after lift-off. Even with increased laser intensity, no auto fluorescence could be detected on the sample subsequent to lift-off, again demonstrating the efficient removal of the PR layer upon NMP treatment.
Characterization and optimization of the MAPL process

Figure 4.3  The left fluorescence image shows squares of 60x60 μm² of niobia (black) separated by a 1.7 μm thick photoresist. Autofluorescence of the PR was observed when excited at a wavelength of 488 nm (longpass filter 505 nm). The image on the right shows the sample after lift-off, no fluorescent signal was detected.

4.1.2 PLL-g-PEG adlayer before and after lift-off

4.1.2.1 XPS RESULTS

We were interested in measuring the stability of the polymer during the lift-off process. Such measurements are relevant with respect to potential loss of functionalized polymer as well as protein resistance of the adlayer. Niobium-oxide-coated silicon chips were homogeneously coated with PLL-g-PEG and analyzed by XPS before and after lift-off. Detail scans of C1s and Nb3d were fitted in order to evaluate the ratios of C1s (C-O) to Nb3d intensity (sum of both spin-orbit components, i.e. 3d_{3/2} and 3d_{5/2}). Such ratio provide information on the polymer surface coverage since spectral contribution from C(C-O) is primarily due to PEG and Nb to the substrate. Our aim was to compare these ratios before and after lift-off. The intensities measured with the XPS Sage instrument can readily be ratioed and compared since this instrument has a linear behavior between the ad-hoc ratios and the modeled layer thickness (personal communication by Laurent Feuz, LSST-ETHZ).
Table 4.2  Intensity ratios of C1s (C-O) over Nb3d of PLL-g-PEG coated niobia surfaces before and after lift-off in NMP.

<table>
<thead>
<tr>
<th></th>
<th>PLL-g-PEG</th>
<th>PLL-g-PEG+lift-off</th>
</tr>
</thead>
<tbody>
<tr>
<td>I C1s (PEG) / I Nb3d</td>
<td>0.168</td>
<td>0.177</td>
</tr>
<tr>
<td>standard deviation</td>
<td>0.010</td>
<td>0.016</td>
</tr>
</tbody>
</table>

As shown in Table 4.2 the differences between the surface before and after lift-off are not significant with respect to the calculated standard deviation (n=4).

Figure 4.4 shows the detail spectra of C1s signal before and after lift-off. The C1s peak was fitted with 3 components and the area of the peak C-O,C-N was used for the ratio calculation as presented above.

![Figure 4.4 Detail spectra of C1s signal of PLL-g-PEG on niobia before and after lift-off. Each C1s peak was fitted with 3 components, namely C-C (285.0 eV); C-O,C-N (286.6 eV) and C=O (288.5 eV).](image)

Both C1s peaks (before and after lift-off) were very similar indicating that the monomolecular coating was not altered in a detectable manner. Since the differences, if any, appear to be very small, one can argue that XPS is not sensitive
enough for properly detecting the surface changes at this level. The polymer stability during lift-off has been investigated on other oxides and published by Städler et al. (diploma thesis 2003, LSST-ETHZ). We found that XPS could clearly demonstrate the loss of PLL-g-PEG when initially adsorbed on titania or silica surfaces. Significant amounts of polymer were desorbed during the lift-off process on both of these oxides. Based on these observations, we decided to choose niobium oxide as the preferred substrate.

4.1.2.2 OWLS RESULTS

Optical waveguide lightmode spectroscopy (OWLS) was used as a complementary technique to gain additional information on the potential polymer desorption in the organic solvent. This technique, described in chapter 10, provides in situ readout of adsorbed masses on waveguides (coated in this case with Nb₂O₅). It was also used to test the protein resistance towards serum.²⁰⁵-²⁰⁷

PLL-g-PEG and its functionalized variants (biotin and peptide) were adsorbed on O₂-plasma-cleaned, niobia-coated waveguides and tested for protein resistance. Adsorbed masses for the three polymers used in this thesis are presented in Table 4.3. The dn/dc (i.e., the increment of refractive index with concentration) used for the mass calculation was 0.158 cm³/g.²⁰⁸
Table 4.3  Adsorbed mass of the different polymers on niobium oxide calculated from OWLS data using a dn/dc of 0.158 cm$^3$/g. Errors represent standard deviations on minimum four measurement per polymer. The calculation of the ligand surface density based on OWLS results is detailed in chapter 10, equation 10.4 and equation 10.5 for mixed coatings.

<table>
<thead>
<tr>
<th>adsorbed polymer mass [ng/cm$^2$]</th>
<th>PLL-g[3.3]-PEG</th>
<th>PLL-g[4.1]-PEG/PEG-biotin</th>
<th>PLL-g[3.7]-PEG/PEG-RGD</th>
</tr>
</thead>
<tbody>
<tr>
<td>average polymer Mw [kDa]</td>
<td>68</td>
<td>74</td>
<td>65</td>
</tr>
<tr>
<td>(not needed for calculations)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ligand surface density [pmol/cm$^2$]</td>
<td>-</td>
<td>26.6±3.9</td>
<td>4.8±0.6</td>
</tr>
<tr>
<td>ligand surface density [μm$^2$]</td>
<td>-</td>
<td>165’000±25’000</td>
<td>28’000±3’600</td>
</tr>
</tbody>
</table>

Lift-off conditions were simulated in the OWLS flow chamber in order to gain additional information on the polymer adlayer stability in contact to NMP. Nio-bia-coated waveguides were coated in situ with, either the RGD-modified or the biotin-functionalized polymer. The lift-off process was then simulated by injecting 1 ml of ultrapure water followed by 4 injection of 1 ml of NMP resulting in a 4 min incubation in the organic solvent. In certain cases, the polymer was reinjected in order to identify a potential "surface healing" mechanism.

In total ten experiments were performed and an average polymer loss of 13 ng/cm$^2$ was measured with a standard deviation of 9. No significant differences were observed between the two types of polymers (biotin or peptide functionalized). Figure 4.5 is a typical OWLS curve of this serie of experiments; upon adsorption of functionalized PLL-g-PEG and Hepes 2 rinsing, a polymer mass of 152 ng/cm$^2$ was measured. After 5 min of incubation in NMP and subsequent buffer rinsing a loss of 14 ng/cm$^2$ was observed. As mentioned earlier, the stan-
standard deviation over the 10 experiments was rather large (9) and we selected this example because it is the closest to the average value. In some experiments no loss was observed, while in other cases larger desorption were occurring.

A second polymer injection results in a polymer re-adsorption and recovery of the adlayer. Complete recovery was not achieved in all experiments. Interestingly, the apparent loss of polymer measured with OWLS was not detected by XPS measurements. In an attempt to further clarify this point we performed ellipsometry measurements.

![Figure 4.5 OWLS curve showing PLL-g-PEG/PEG-biotin subjected to NMP followed by polymer re-injection.](image)

**4.1.2.3 Ellipsometry (ELM) results**

Niobia-coated wafers (1x1 cm²) were O₂ plasma cleaned and immediately measured with ellipsometry (ELM) to determine the oxide thicknesses of each sample. The same samples were then coated with PLL-g-PEG and their thickness
determined. Subsequently, the lift-off protocol was performed on these samples and measured again. Table 4.4 documents the averaged thicknesses of polymer before and after NMP lift-off. Note that the absolute thicknesses should be considered with caution since this experiments were not performed under controlled relative humidity.

<table>
<thead>
<tr>
<th>Table 4.4</th>
<th>Polymer coating before and after lift-off.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PLL-\text{-}g\text{-}PEG</td>
</tr>
<tr>
<td>thickness [Å]</td>
<td>12.9±0.7</td>
</tr>
</tbody>
</table>

The ELM measurements, performed on 5 samples, demonstrated a small loss of polymer. However, thickness values among samples measured after lift-off showed a significant scatter (standard deviation of 2.1). The average thickness reduction of 1.2 Å lies within the uncertainty of the ELM technique. It is interesting to note that when the PLL-\text{-}g\text{-}PEG powder is dissolved in NMP, the same polymer thicknesses were measured, implying that the polymer can be adsorbed out of the organic solvent onto the oxide surfaces. Other solvents were tested by Markus Müller (LSST-ETHZ); he was able to adsorb the polymer from ethanol solution with similar surface coverage to those in standard buffer (Hepes).

4.1.3 Polymer exchange at the surface

Potential exchange of surface-immobilized PLL-\text{-}g\text{-}PEG with biotin-functionalized PLL-\text{-}g\text{-}PEG added in solution was addressed via OWLS.

The MAPL patterns are constituted of functionalized (the adhesive patches) and non-functionalized polymer (the background). Since the process is carried out in a stepwise manner the functionalized polymer is already immobilized on the surface when the second, non-modified polymer, is adsorbed onto the back-
ground. We investigated the potential surface exchange between these two polymers. To visualize such an effect, we first adsorbed PLL-g-PEG in situ on a niobia coated waveguide that we then exposed for 30 min to a solution of biotinylated PLL-g-PEG (deliberately at the higher concentration of 1 mg/ml). Upon buffer rinsing we measured the adsorption of streptavidin (20 µg/ml in H2 for 20 min). A dn/dc value of 0.182 cm³/g was used to calculate the mass of bound streptavidin. Figure 4.6 is a typical OWLS curve of this experiment.

**Figure 4.6** OWLS curve showing exchange of PLL-g-PEG by PLL-g-PEG/PEG-biotin and subsequent streptavidin adsorption.

On average 40±11 ng/cm² of bound SA were measured on the biotinylated surfaces. Since less than 2 ng/cm² of SA adsorbed on PLL-g-PEG (data not shown) we can calculate the average amount of PLL-g-PEG/PEG-biotin needed to bind 40 ng/cm² of SA. A homogeneous adlayer of the same biotinylated polymer (165
ng/cm²) adsorbed 255±4 ng/cm² of SA. Based on these values we can calculate the equivalent mass of biotinylated polymer. We found, if the system follows a linear behavior, that 26 ng/cm² of biotinylated polymer are required to specifically bind to 40 ng/cm² of SA.

These results show that exchange of polymers took place within a short time (30 min with 1 mg/ml). It is likely that this exchange is time-dependent and that it would probably be more pronounced with longer incubation times and higher concentrations.

4.1.4 Discussion on the characterization of homogeneous surfaces

XPS and fluorescence measurements demonstrated that the photoresist was thoroughly removed when the optimized protocol was applied. This step is critical since any surface residues, even a fraction of an atomic monolayer, is likely to inhibit the adsorption of the PLL-g-PEG. The second critical aspect during the lift-off process is the loss of polymer. XPS and ELM data suggested that no significant amounts of polymers desorbed in the NMP, while OWLS measurements indicated an average loss of 13 ng/cm². Large variations in polymer loss were recorded across the ten experiments. These differences have to be judged by taking into account the relatively large standard deviation (9) with respect to the average loss value (13). In certain cases no loss of polymer was recorded while in others a significantly lower polymer mass was measured after NMP incubation (independently if the polymer was biotin or peptide functionalized). When polymer was subsequently re-injected, an increase in mass was generally measured; however, here again, inconsistencies were observed; in certain cases, complete "surface healing" was observed, meaning that the same mass was reached as before NMP injection, while in other cases only partial recovery occurred. The
Characterization and optimization of the MAPL process

fact that a "surface healing" mechanism is occurring suggests that free spaces were available for additional molecules.

Based on these results we cannot make a strong statement whether some polymer was lost during lift-off or not. However, if we only consider the results based on OWLS, where an average loss of 13 ng/cm² was reported, we can safely say that maximum 8% of a full monolayer was lost during lift-off. Also, it is worth noting that 8% are smaller than the uncertainty established for the ligand surface density and therefore falls within the final uncertainty margin.

A loss of polymer coverage could arguably lead to an increased amount of non-specifically adsorbed proteins. Two arguments support that this is not the case. First, it has been shown that the protein resistance of the PLL-g-PEG system depends on the EG monomer density. Even if 13 ng/cm² were desorbed from the niobia, the resulting EG surface density would still be sufficient to largely inhibit the adsorption of serum proteins. To bring this aspect in another perspective we can compare our results with the mass of PLL-g-PEG adsorbed on TiO₂ coated waveguide. The mass of polymer adsorbed on titanium oxide is on average 157 ng/cm² (when calculated with dn/dc = 0.158) as reported by Tosatti et al. and this surface still resists adsorption of proteins. Secondly, the fact that a "surface healing" mechanism is occurring upon polymer re-injection results in an increased EG monomer surface density.

With the polymer exchange experiment performed in the OWLS instrument we have seen that biotinylated polymer in solution can replace surface immobilized PLL-g-PEG. In the MAPL process the order is inversed; we first immobilize the biotinylated polymer and then backfill with PLL-g-PEG. We have no evidence of exchange occurring in that situation due to our inability to perform such a measurement with enough sensitivity. However, if such a mechanism is taking place it will be less dramatic than in the presented model experiments. The
consequences of such events would "only" lead to a reduction of the ligand density but would not harm the protein resistance properties of the adlayer. Moreover, it is important to keep in mind that we performed the experiment at 1 mg/ml even though the MAPL process is carried out at 0.1 mg/ml. It is reasonable to suppose that the exchange occurs to a smaller extent when the concentrations are decreased.

All these arguments tell us that the step-wise procedure selected for the MAPL method is adequate: First, the functionalized polymer is adsorbed and secondly PLL-g-PEG is used for backfill. At the beginning of this PhD project the opposite approach was chosen by using an image reversal PR (negative image, Ti35 PR) resulting in small islands of PR and a background of niobia (note that this can also be done by designing a "negative" mask). With this approach the passivating background was adsorbed first and the functionalized polymer adsorbed at the end of the process. This strategy was not further followed in view of the above arguments as well as the observation that the Ti35 photoresist was more difficult to remove completely (however, this could be optimized if necessary for future applications).

4.2 Characterization of micropatterns

The basic parameters of the MAPL process such as type of photoresist and lift-off solvent, and their associated processing parameters, were established on the homogeneously coated surfaces (section 4.1). Additional surface analysis techniques were employed to precisely monitor each individual assembly step of the MAPL process: Optical microscopy to assess the photoresist patterns quality directly in the clean room; scanning atomic force microscopy (imaging AFM) to evaluate the thickness of the adsorbed polymeric adlayer as well as the spatial
fidelity of the polymer pattern with respect to the photoresist mask. To characterize the spatial distribution of the various chemical compounds, time-of-flight secondary ion mass spectrometry (ToF-SIMS) was used in both, imaging and spectral mode, while the patterns, after exposure to proteins and cells, were characterized by confocal laser scanning microscopy (CLSM).

### 4.2.1 Optical microscopy of photoresist-niobia patterns

A number of parameters directly influence the quality of the pattern transfer from the mask into the photoresist. The final resolution of the structures in the PR is mainly dependent on the chromium mask resolution, the level of residual solvent in the PR (soft-baking), the contact quality between the mask and the PR-coated substrate, the illumination dose as well as the development time and agitation. The quality of the photolithography process was verified for each wafer directly in the clean room using an up-right optical microscope. The optimized photolithography processing parameters can be found in the chapter 10. The resolution limit of standard photolithography is 1-2 µm. When such resolution was desired we had to slightly adapt the standard protocol. By diluting the PR with the proper solvent we could reduce the PR thickness from 1.7 µm to 400-500 nm and thus achieve 1 µm wide lines separated by 1 µm.\textsuperscript{156} However, such high resolution patterns were difficult to reproduce over the whole 100 mm diameter wafer. All results presented in this thesis were achieved with non-diluted PR unless specifically mentioned.

Figure 4.7 shows two different patterns of photoresist after development. Image a) is a square pattern of 60×60 µm\(^2\). The summits are not perfectly at 90° but are slightly rounded. This illustrates that the pattern transfer from the mask to the PR cannot be achieved with high resolution unless the PR is diluted and con-
sequently thinned. Note that three additional parameters play a critical role on the pattern resolution i.e.; softbake time will determine how much solvent is removed from the spin-coated resist and thus influence pattern quality; the illumination dose (energy) has to be within a processing window; finally the development time has to be optimized in order to remove the illuminated PR but without over developing the structures and loosing the sharp edges. The small magnification image (b) is used to illustrate that photolithography allows the patterning of thousands of features at the same time with virtually no defects over cm² areas.

![Pattern contrast of photoresist and niobium oxide substrate](image)

**Figure 4.7** Pattern contrast of photoresist and niobium oxide substrate a) 60x60 μm² square. b) round patterns, diameter 20 μm, pitch 120 μm.

### 4.2.2 Imaging AFM

The mask patterns used for this investigation were lines of 2 μm width with a pitch of 4 μm. To achieve this resolution level, the PR was thinned by an organic solvent (see chapter 10). MAPL samples were produced up to stage III (Figure 3.2) and analyzed with imaging AFM, tapping mode in air. The lines of assembled PLL-g-PEG/PEG-biotin are unambiguously identified in the AFM image of Figure 4.8a. At the higher magnification the pattern edges appear sharp
and thus show a high spatial fidelity (Figure 4.8b). The bare Nb₂O₅ background is smooth and no inhomogeneity were observed suggesting that the PR has been either completely removed or that an ultrathin homogeneous layer (typically a monolayer) remains on the surface. This question is addressed later with ToF-SIMS.

The AFM topography line scan in Figure 4.8 across the stripes pattern shows a regular and well defined profile with an average step height of approximately 1.5 nm; this value is fairly consistent with the "dry" layer thickness determined in air by spectroscopic ellipsometry (Table 4.4). However, this value is to be considered with caution since it is well known that thickness measurement of organic thin films with AFM tapping mode is extremely sensitive to the instrumental parameters. Also, these data were recorded without taking into account the environment relative humidity, which is known to influence the degree of swelling of this PEG brush polymer. The important information here is that only one monolayer is adsorbed on the surface. This aspect is essential for the controlled immobilization of ligands. Multilayers architectures are generally less well defined in terms of ligand surface density than monolayers with bioactive groups exposed and presented at a flat surface.

Upon backfill with PLL-g-PEG the patterns were still visible and a residual step height of approximately 0.5 nm was recorded (data not shown). This remaining topography is attributed to the longer PEG chains (3.4 kDa vs. 2 kDa) that carry the biotin groups in the functionalized copolymer.
4.2.3 ToF-SIMS analysis

ToF-SIMS was used in both, spectral and imaging mode. Spectra were acquired to identify fragments specific to the different chemistries on the surface (PR; PLL-g-PEG and niobium oxide). Imaging was useful to determine the surface distribution of the different chemical components involved in the MAPL process. Note that ToF-SIMS is a highly surface sensitive technique compared to XPS since the sampling depth of 1-2 nm is very shallow. 203, 210

Figure 4.9 shows a positive spectrum of the photoresist (homogeneous PR layer). Two fragments specific to the PR with m/z values of 121.09 and 147.04
were identified. The fragment corresponding to \( m/z \) 121.09 (C\(_8\)H\(_9\)O\(^+\)) is attributed to the novolak-resin present in the PR.\(^{211}\) The fragment of \( m/z \) 147.04 cannot be attributed to any of the known components of the PR (see chapter 10). We believe that this fragment originates from R group (not published) bound to the photoactive compound. The peak at \( m/z \) 149 (C\(_8\)H\(_5\)O\(_3\)\(^+\)) is attributed to contamination originating from a plasticizer used for the production of sample containers.\(^{212}\)

![Figure 4.9](image-url)  

**Figure 4.9** ToF-SIMS positive spectra of S1818 photoresist. The fragments specific to the PR are marked with arrows in the insert.

The situation is much clearer with the fragments originating from either the PLL-g-PEG or the oxide substrate. The fragments specific to PLL-g-PEG and niobia are indicated on the spectra in Figure 4.10 and listed in Table 4.5. Note that the fragments specific to PLL-g-PEG were published earlier by Stéphanie Pasche (LSST, ETHZ).\(^{91}\)
Figure 4.10 ToF-SIMS positive spectra of PLL-g-PEG on a niobia surface. The fragments specific to PLL-g-PEG and to the niobia substrate are indicated on the spectrum. The Cs$^+$ peak is caused by the detection of previously implantation primary Cs$^+$ ions.

Table 4.5 Positive fragments used for this study.

<table>
<thead>
<tr>
<th>source</th>
<th>fragment</th>
<th>m/z</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLL</td>
<td>$\text{C}_3\text{H}_6\text{N}^+$</td>
<td>56.05</td>
</tr>
<tr>
<td>PLL</td>
<td>$\text{C}<em>5\text{H}</em>{10}\text{N}^+$</td>
<td>84.08</td>
</tr>
<tr>
<td>PEG</td>
<td>$\text{C}_4\text{H}_7\text{O}^+$</td>
<td>71.05</td>
</tr>
<tr>
<td>PEG</td>
<td>$\text{C}_3\text{H}_5\text{O}_2^+$</td>
<td>73.03</td>
</tr>
<tr>
<td>PEG</td>
<td>$\text{C}_4\text{H}_9\text{O}^+$</td>
<td>73.07</td>
</tr>
<tr>
<td>PEG</td>
<td>$\text{C}_4\text{H}_9\text{O}_2^+$</td>
<td>89.06</td>
</tr>
<tr>
<td>niobia</td>
<td>Nb$^+$</td>
<td>92.8</td>
</tr>
<tr>
<td>niobia</td>
<td>NbO$^+$</td>
<td>108.9</td>
</tr>
<tr>
<td>PR S1818</td>
<td>$\text{C}_8\text{H}_9\text{O}^+$</td>
<td>121.09</td>
</tr>
<tr>
<td>PR S1818</td>
<td>not known</td>
<td>147.04</td>
</tr>
</tbody>
</table>
These specific fragments were then mapped by imaging mode at different stages of the MAPL process. The images in Figure 4.11 were based on the specific fragments of PLL-g-PEG, niobia or PR: Image a) demonstrates that the PLL-g-PEG is localized in the patterns, while the background presents few counts only. Images b) and c) show, respectively, an intense signal related to the niobia substrate and a noise-type signal for the PR specific fragments. In both cases the patterns show less intensity due to shadowing by the PLL-g-PEG adlayer. The signal recorded for the photoresist is barely detectable since the highest intensity pixels (the yellow ones) correspond to 3 counts only. The surface after PLL-g-PEG background passivation shows a homogeneous coverage (image d). This suggests that similar amounts of polymer adsorbed inside the patterns and in the background.
Figure 4.11 Imaging ToF-SIMS of the surface after lift-off (a,b,c) and after PLL-g-PEG backfill (d). Each picture is based on the sum of positive specific fragments of a) PLL-g-PEG (m/z: 56.05; 76.05; 73.03; 73.07; 84.08; 89.06), b) niobia (m/z: 92.9; 108.9), c) photoresist (m/z: 121.09; 147.04), d) PLL-g-PEG (m/z: 56.05; 76.05; 73.03; 73.07; 84.08; 89.06).

4.2.4 Streptavidin patterns studied with CLSM

Confocal laser scanning microscopy was used to visualize biotin patterns decorated with streptavidin alexa-conjugated fluorescent dyes. Figure 4.12 shows various pattern shapes on relatively large areas. In both images the patterned surface is homogeneous and very little defects were observed. Such results demon-
strate the powerful advantage of applying a parallel and highly reproducible process such as photolithography. Figure 4.12a. shows a surface patterned with "biohazard signs" that only serves the purpose of illustrating that virtually any pattern geometry can be achieved by combining photolithography and MAPL. This micropattern quality can consistently be achieved by carefully following the protocol developed in the frame of this thesis.

![Figure 4.12 PLL-g-PEG/PEG-biotin patterns decorated with alexa-488 labeled streptavidin](image)

**Figure 4.12** PLL-g-PEG/PEG-biotin patterns decorated with alexa-488 labeled streptavidin a) "biohazad signs" b) 20 μm round dots. Both on a niobia-coated substrate.

The MAPL technique was tested on other substrate surfaces, namely TiO₂, SiO₂ and indium-tin oxide (ITO). The pattern homogeneity was as good as on the Nb₂O₅-coated substrate. However, the important difference between niobia and the other oxides is that a significant amount of polymers were lost during lift-off step (Section 4.1.2.1). This results in a reduced ligand surface density and consequently in a lower fluorescent signal. The aspect of the bioligand density on niobia-coated surfaces in addressed in the next section.
4.2.5 Micropatterns with controlled ligand density

One of the major motivations for developing this novel patterning technique was to produce functional micropatterns with controlled surface density of bioligands while preserving the non-fouling properties. These two aspects are essential for inducing highly specific and quantifiable interactions with the biological milieu. The capability of the MAPL technique to produce patterns with controlled bioligand densities independent of the pattern geometry is demonstrated in this section. A series of 5 samples with decreasing biotin surface densities (within the spots) were produced by mixing appropriate solution volumes of PLL-g-PEG and PLL-g-PEG/PEG-biotin and assembling from such solutions (stage II, Figure 3.2). The molar mixing ratios of PLL-g-PEG/PEG-biotin to PLL-g-PEG were respectively, 100/0, 50/50, 20/80, 10/90 and 0/100. The fully processed samples were exposed to a solution of labeled streptavidin (20 µg/ml in Hepes 2 buffer) for 30 min and rinsed with Hepes 2, ultrapure water and finally N₂ blow-dried. The CLSM images of the patterns produced with each of the five mixed polymer solutions are shown in Figure 4.13. The decrease in fluorescent signal in the spots is clearly visible in the corresponding series of images. The control pattern in e) contains no biotin because non-functionalized PLL-g-PEG was adsorbed in both steps (spot and background). The uniformly dark background demonstrates the excellent resistance of the MAPL interfacial architecture to non-specific adsorption and the absence of noticeable defects in the final polymeric adlayer. The fluorescent intensity values were quantitatively investigated on each surface. First, the dynamic range of the microscope was set with the detector gain, controlled by the PMT (upper limit), and amplifier offset (lower limit). The sample displaying the highest fluorescent intensity was used to set the upper limit but carefully avoiding any signal saturation. The sample coated with PLL-g-PEG only was bleached in an area of interest (AOI) with
1'000 iterations (i.e. each pixel was illuminated 1000 times with 100% laser power to "burn" the fluorescent label) and set so that the bleached region corresponds to a signal of ~5 (background).

Figure 4.13 CLSM images of 20 μm diameter spots of PLL-g-PEG/PEG-biotin in a PLL-g-PEG background. The biotin spots are decorated with fluorescent labeled streptavidin: a-e) The biotin surface density was varied through assembly from mixed aqueous solutions of biotin-functionalized and non-functionalized PLL-g-PEG in different, descending molar ratios of the two polymers. f) Depicts the streptavidin fluorescence intensity versus the calculated biotin surface density based on OWLS measurements.

The inserts represent intensity line-scans over four spots. In each serie one spot was bleached repeatedly to completely "de-activate" any fluorophore in the light path. The line scans provide two interesting informations. Firstly, the intensity where the spot was bleached is identical to the intensity of the PLL-g-PEG background. This tells us that no non-specifically adsorbed SA could be detected. Secondly, the intensity across individual spots is rather homogeneous. Also, the average spot intensities across several spots showed relatively small variations.
These small dissimilarities were quantified as follows: the fluorescence intensity was measured on each sample on several spots and then averaged. On each spot the standard deviation was recorded and then averaged over several spots (for further detail see Section 7.2). These average standard deviation values were reported as error bars on the fluorescent intensity graph (f). The abscisse in graph (f) represents the biotin surface densities inside the spots (pmol/cm²). These values were calculated based on the adsorbed polymer mass (165±10 ng.cm⁻²) measured by OWLS, the grafting ratio (g = number Lys monomers per PEG chain = 4.1), the percentage of functionalized PEG chains (x=50%) on the biotinylated polymer and the mixing molar ratio (y). All the equations and values used are listed in chapter 10; the results are presented in Table 4.6.

Table 4.6 Calculated biotin surface densities for different mixing ratios

<table>
<thead>
<tr>
<th>molar mixing ratios PLL-g-PEG/PEG-bio / PLL-g-PEG</th>
<th>100:0</th>
<th>50:50</th>
<th>20:80</th>
<th>10:90</th>
<th>0:100</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biotin surface density [pmol/cm²]</td>
<td>26.6</td>
<td>13.3</td>
<td>5.3</td>
<td>2.7</td>
<td>0</td>
</tr>
<tr>
<td>Biotin surface density [µm⁻²]</td>
<td>165'000</td>
<td>83'000</td>
<td>33'070</td>
<td>16'500</td>
<td>0</td>
</tr>
</tbody>
</table>

The streptavidin fluorescence intensity was found to be proportional to the biotin surface density in the lower concentration range (<10 pmol.cm⁻²) but leveled-off at the higher biotin surface densities. This plateau was attributed to surface saturation of streptavidin molecules. We are confident that surface saturation is reached based on the biotin density calculation which indicates that the density is 0.17 biotin/nm² (=1 biotin/5.8 nm²). The size of a SA molecule was reported by Darst et al.¹²¹³ to be 5.5x4.5 nm² i.e. one SA occupies 25 nm² which is significantly larger than 5.8 nm². A theoretically perfect monolayer of SA represents a
Characterization and optimization of the MAPL process

surface density of 7.26 pmol/cm$^2$. This value does not $a$ priori correlate with our OWLS measurements where 4.8 pmol/cm$^2$ (255±4 ng/cm$^2$) of SA adsorbed on a monolayer of PLL-g-PEG/PEG-biotin (50%) on Nb$_2$O$_5$. The difference can have multiple origins; firstly, a maximum packing of a protein in a monolayer is unlikely because the protein cannot be accommodated on the whole surface without any gaps in between them. Furthermore, SA carries a small net negative charge which can locally induce electrostatic repulsions and hence increase the distance between the proteins.

The MAPL process has been applied to other substrates such as TiO$_2$, SiO$_2$ and ITO (ref clarence and jost thesis or paper) (indium-tin oxide). The geometries of the PR patterns could be translated into SA patterns with the same fidelity as on niobia. However, the lower fluorescence intensity observed on all substrates compared to niobia confirms the loss of functional polymer during the lift-off process. Note that on ITO the reduction of intensity can also be caused in part by quenching of the fluorophores since it is an electrically conducting surface.

The substrate format generally used in this study was 1x1cm$^2$ samples that where cut out of a 100 mm diameter wafer. However, depending on the use of the pattern it may also be directly performed on standard microscopy slides or on light guiding surfaces for new experimental setup such as the Waveguide Excited Fluorescence Microscope (WExFM; Michelle Grandin, LSST, ETHZ). Note that one limitation of the MAPL process is that the surface density of bioligands is the same in each island. A summary on the pattern characterization and future developments can be found in chapter 9 (Conclusions and outlooks).
This chapter presents results of various cell types on MAPL patterns. The attachment of the cells on large patterns as well as single cell arrays are discussed. Cell adhesion strength differences between a fibronectin coating and a synthetic peptide surface are reported. Finally, issues of pattern defects and pattern stability are addressed.

Four different cell lines were used in this work, namely human foreskin fibroblasts (HFF); mouse 3T3’s fibroblasts (expressing GFP-labeled β3-integrin); murine NIH 3T3’s fibroblasts and MG63 human osteosarcoma cells. Details on these cells and culture conditions can be found in chapter 10. The reason to work with several cell lines was mainly dictated by the access to cell lines at the different research locations: the work performed at ETHZ was with HFF and 3T3 mouse fibroblasts, while the work performed at Georgia Tech was with MG63 and NIH 3T3’s fibroblasts. On the other hand, the goal was to demonstrate that the MAPL surfaces are versatile platforms for fundamental cell studies that can potentially be applied for a large library of adherent-cell types.

Patterns were rendered cell-adhesive with PLL-g-PEG/PEG-RGD, either pure or mixed with PLL-g-PEG if smaller RGD densities were desired. The amount of
RGD per unit area on niobia of a non-mixed solution are calculated based on OWLS data and polymer architecture; they can be found in Table 4.3. Note that all experiments were conducted in 10% sera-containing media and that unattached cells (on the non-adhesive coating) were removed by media exchange four to five hours after seeding (gently aspirated with a hand-micro-pipette rather than vacuum suction).

5.1 Locally confluent cultures on "large" patterns

A variety of shapes and sizes of RGD-peptide patterns were produced in the frame of this thesis. In this paragraph, surfaces with islands large enough to accommodate multiple cells are presented. Figure 5.1 shows that fibroblasts attached and spread specifically on the peptide-functionalized areas. This demonstrates that the PLL-g-PEG background is non-adhesive for the cells and hence inhibits the growth, out the peptide patches. The functionalized squared patterns used had dimension of 200×200 μm² (40’000 μm² per patch, a) and 60×60 μm² (3’600 μm² per patch, b and c) and had 28’000 RGD/μm². For both types of patterns, each island was generally occupied by multiple cells however, on the 60x60 μm² some patterns were either occupied by no cell or only one as seen in Figure 5.1c. The cell in Figure 5.1c was stained for vinculin, a protein clustering in focal adhesions (FA). The FA are clearly identifiable as the green elongated dots, mostly at the cell periphery. They are typical of spread cells having an organized cytoskeleton. In a first approximation, this demonstrates that the PLL-g-PEG/PEG-RGD supports fibroblast spreading and attachment (FA) in a similar way as traditional substrates used in cell culture.

By culturing the fibroblasts for several days we could observe that, on such
patterns, the cells kept proliferating despite of the geometrical spreading constraints. For a given pattern, the number of cells increased with culture time. For long time culture and pattern stability we refer the reader to Section 5.3. Two types of negative controls were performed to ensure that the cell-receptors specifically interacted with the RGD sequence of the peptide and not with unspecifically adsorbed proteins. In the first case, PLL-g-PEG without the RGD motive was immobilized in both adsorption steps, resulting in a homogeneous PLL-g-PEG coating. No cells were able to attach demonstrating that non-specific protein adsorption from serum does not take place, to a degree sufficient to sustain cell spreading. Secondly, the specificity of the RGD motive was tested with a polymer functionalized with the scrambled sequence: RDG. Very few cells weakly attached to the surface but did not spread as already shown in earlier publications using the same type of functional polymers.\(^{48,160}\) Moreover, these cells were usually washed away during media exchange.
Cells on patterned surfaces were cultured for up to 2 weeks in order to investigate cell viability. Media was changed every 2 days and the amount of detached cells (floating in the suspension) was comparable to a standard culture of the same cells on tissue culture polystyrene (TCPS). The attached cells displayed no abnormalities in terms of cell spreading i.e., no significant footprint changes were observed. Live-dead assays confirmed that not more apoptotic cells were
observed on MAPL surfaces than on tissue culture polystyrene (TCPS) (with MG63 cells). The absence of toxic effects, that could result form residues from the sample processing, demonstrates that the MAPL surfaces are suitable for in vitro cell culture for at least two weeks.

Cells were also cultured on highly anisotropic patterns in terms of geometry. MG63 cells were seeded on RGD lines of different width in order to investigate to which degree the MAPL patterns can constrain and direct cell spreading and hence, cell shape. All line widths (10, 20, 30 µm with 100 µm spacings) sustained cell attachment and forced the cells to spread along the RGD lines. On 10 µm wide lines we observed that each cell was in contact with maximum two cells (one on each side) as shown on Figure 5.2 a and b. Even under these spreading conditions the cells were still able to proliferate as the increase in cell density after 72 h demonstrates (image c). Cellular patterned cultures with either no or a controlled number of cell-cell contacts have been shown to be useful for answering a number of questions related to cell-cell communications and hence, cell proliferation. Organized cell assemblies of only one, or a few cells, are highly challenging in terms of surface patterning and will be discussed Section 5.2.
Figure 5.2  MG63 on PLL-g-PEG/PEG-RGD lines of 10 \( \mu \text{m} \) width, spaced by 100 \( \mu \text{m} \). a) and b) were taken 24 hours after seeding, c) after 72 hours.

Figure 5.3 shows the versatility of the MAPL technique to produce complex pattern geometries with cells.

Figure 5.3  DiI-membrane stained fibroblast revealing the underlying RGD pattern (MAPL letters).
The negative controls presented above (RDG and pure PEG surfaces) suggest that specific interactions between the synthetic RGD-peptides and the cell-receptors take place. To further support this affirmation the OWLS data on PLL-g-PEG/PEG-RGD (Section 3.1) has also been reported to adsorb less than 2 ng/cm$^2$ of serum proteins when immobilized on niobia. This result is important since it tells us that the RGD-containing peptide does not interact with proteins from serum and thus, the only ligand available to the cells is the linear RGD motive. Many studies have been published on the interactions between adherent cells and engineered RGD peptides. Several integrin heterodimers are known to bind to the Arg-Gly-Asp acid sequence, in particular $\alpha_5\beta_1$ and $\alpha_v\beta_3$. The RGD epitope is found in a loop conformation on the 10th type III repeat of fibronectin (FnIII$_{10}$).$^{215}$ Recent results refined our view of these interactions; Garcia and coworkers came to the conclusion that both integrins ($\alpha_5\beta_1$ and $\alpha_v\beta_3$) bind to the RGD motive of Fn, but $\alpha_5\beta_1$ binding also requires the PHSRN synergy site in the 9th type III repeat of Fn.$^{37}$ This suggests that in our case, only the $\alpha_v\beta_3$ integrin is activated and therefore, it is reasonable to hypothesize that the resulting integrin-triggered signaling cascade is likely to have a significantly different pattern than on a fibronectin surface. Many others, also reported differences in cell activity between Fn or RGD surfaces.$^{41,47}$ Note that in Section 5.5 we discuss this issue in more details. To further highlight the complexity of these systems, differences in peptide activity depending on their loop or linear architecture were clearly identified.$^{44,216,217}$

This brief discussion about integrin coupling to the synthetic peptides serves the purpose to underscore that slight changes at the ligand level is likely to affect the cellular behavior. Also the precise analysis of these mechanisms reach beyond the scope of this work.
5.2 SINGLE CELL PATTERNS

Manipulating and studying cells at the individual level is believed to bring significant new insights in fundamental cell mechanisms. The growing numbers of labs/institutes specially dedicated to single cells studies (e.g., Systems Biology, ETHZ in Basel) testify of this innovative but challenging trend.

Two parameters can easily be varied with the aim to reduce the number of cells per island to eventually a single cell. The first is the seeding density; plating cells at very low concentrations (number of cells similar to number of patterns) will result in a fraction of the islands occupied by a single cell only. However, this strategy is limited because the fraction of patterns fulfilling the individual cell requirement might not be satisfying. The acceptable fraction of patterns supporting a single cell are dictated by the experimental requirements. If the cells are to be addressed individually with, for example an optical microscope, then such surfaces might be adequate since one can visually distinguish between multicellular islands vs. singly occupied islands and could thus investigate the latter ones only. This approach is illustrated in Figure 5.4 where 60x60 μm² patterned surfaces were seeded with a) 20’000 cells/ml and b) 7’000 cells/ml. In image a) the stained DNA (blue = nucleus) shows two cells per pattern but only one cell per pattern in b).

Figure 5.4 HFFs on 60x60 μm² RGD islands. a) The patterns are occupied by multiple cells. b) Sample seeded with lower cell density and showing a number of patterns occupied by single cells.
Since the number of cells in the suspension has to be decreased significantly in order to obtain a few islands with individual cells, the resulting surface presents a low number of occupied islands. In several cases this relatively lower number of cells may not be sufficient to get statistically significant data. Of course, one can always produce multiple samples and sum up the measurements; however, this method would not meet the high-throughput capabilities often desired in this field.

A second possible strategy for creating arrays of single cells is to reduce the adhesive island size to physically accommodate only one cell. This approach has the advantage of presenting a rather homogeneous array of single cells throughout the surface as shown in Figure 5.5.

![Figure 5.5](image.png)

**Figure 5.5** A MAPL array of individual MG63 on 20 μm diameter round RGD-islands distant by 50 μm. Image taken 24 hours after cell plating.

The high magnification image in Figure 5.5 (right) also shows islands covered
by two cells. This is because the MG63 cells were still able to proliferate on these 20 μm islands although their spreading was significantly hampered. However, this was not true for every island; about 20% of the MG63 did not proliferate. This proliferation is very clear on Figure 5.6. a) where, cells were cultured for 5 days on 60x60 μm² islands, while b) was after 3 days on 20 μm diameter round islands. In both cases, cell-aggregates are clearly visible.

![Figure 5.6](image)

Figure 5.6  a) MG63 cultured 5 days on 60x60 μm² islands. b) 3 days on 20 μm diameter islands. On both types of patterns the cells did proliferate and grew in columns, away from the surface.

For comparison, MG63 cells on non-patterned RGD surfaces, but of similar surface density, presented projected areas (footprints) of 4600±500 μm², while on the 60x60 μm² and 20 μm diameter patterns their footprint was restricted to, respectively 3600 μm² and 314 μm². In Figure 5.6b, we can see that four cells did not proliferate. We tried to further reduce the adhesive island size to prevent cell proliferation; however, we found that culturing these cells on 10 μm diameter islands was not possible for more than 24 hours; One day after plating the majority of the MG63 cells were detached from the patterns (data not shown). Larger patterns (30 and 50 μm) showed proliferation rates comparable to unpat-
terned surfaces, namely division approximately every 24 hours.

We were also interested to know if the patterned cells were organizing their cytoskeleton similarly to unconstrained cells. Vinculin, f-actin and nuclei were stained in MG63 cells. The complete staining protocols are described in chapter 10. Surprisingly, in order to obtain satisfying stainings in MG63 cells, we had to extensively adapt and elaborate the standard protocol, commonly used for fibroblasts. It was challenging to visualize the actin filaments even in unconstrained cells growing on TCPS. Also, the best results were obtained with low passage numbers, namely 1 to 3. This suggests that these cells may rapidly transform and lose a number of their original characteristics. However, even with low passage numbers we did not observe any stress fibers in patterned cells. As for vinculin, the actin signal was highly diffuse and rather homogeneously distributed. This has also been observed by Thomas et al. reporting no cytoskeleton organization in primary bone cells when patterned on an area smaller than 400 μm². This is not really surprising since it is known that internally generated tensions (by spreading, clustering integrins (FA) and polymerizing actin stress fibers) are crucial for cell survival, proliferation and differentiation. In traditional cell culture (unpatterned) the cells change their morphology from spherical (in suspension) to pancake-like shape. When spreading is sufficiently constrained, the cells keep a rather spherical morphology. This was the case on 20 μm diameter round islands as the profile view in Figure 5.7 exemplifies: The cells had a height (~15 μm) similar to their diameter (20 μm). In such configuration the MG63 cells did not initiate any cytoskeleton organization.
However, other scientists have observed cytoskeleton organization and clear presence of focal adhesions in fibroblasts attached to round islands of 10 μm diameter coated with fibronectin. The puzzling fact with MG63 is, despite their lack of organized internal structure, they were still proliferating. This behavior is in contradiction with what is commonly accepted and published. For example, Chen et al. cultured capillary cells on Fn islands of 10 and 20 μm diameter and observed that the cells remained in a quiescent state (no growth) and hence, did not proliferate. The probable explanation to the unexpected behavior of the MG63 cells lies in their original tissue. These cells were originally taken from a human bone cancer (osteosarcoma) and are thus, transformed (naturally) compared to "healthy" cells. It is known that cancer cells behave differently and that they are less likely to undergo apoptosis under harsh conditions. For example, it is possible to keep them alive on TCPS for at least one week in media without serum. Upon addition of serum (7 days later) these cells would start to proliferate as normally (personal communication from Gary Seeba, Georgia Tech). This also helps explaining the negligible apoptosis rate of these cells on patterns as well as on TCPS. For example, endothelial cells have an apoptosis rate of 20% and 7% when cultured on 10 and 20 μm diameter islands.
respectively, compared to unpatterned substrata. Moreover, it is known that during transformation of a healthy cell into a malignant cell, there is a progressive loss of shape-dependent regulation that may lead to cell survival in the absence of ECM extension. This is usually accompanied by unrestricted mass expansion and disorganized tissue formation. Literature on MG63 cells is rather abundant but often contradictory. Interestingly, despite the large number of publications, it is rare to find any figure showing cytoskeleton staining for this type of cells. Zinger et al. reported that the cytoskeleton organization in MG63 is a time dependent process. In our study we did not see any differences between day 1 and day 4. Yamagata and Kimata hypothesized that the disorganized microfilaments observed in these cells is due to the production of chondroitin sulphate proteoglycans, an anti-adhesive molecule inhibiting the formation of organized stress fibers and focal adhesion. Consequently, this reduces the cell-substrate binding strength and may play a role in metastasis.

In conclusion, we suggest that the MG63 cell line is not an adequate model to study cell-surface interactions out of the frame of cancer research. Also, based on literature and experience, it is reasonable to say that the results are generally closely related to the cell type. The example of mouse fibroblasts forming an actin network on the 10 µm patterns and the rat calvaria osteoblasts showing no organization, is representative of this complexity.

Immobilizing synthetic peptides in a highly controlled manner provide well defined model surfaces for the investigation of ligand density and type on the cellular behavior. However, in certain applications the simple presence of cell-adhesive patches of unspecifically adsorbed proteins, in a non-fouling background is sufficient. We were interested to test if the MAPL process could achieve similar patterns as the SMAP technique or the direct stamping of proteins (micro-contact printing + PLL-g-PEG backfill). We replaced the RGD-functionalized
polymer adsorption (stage II, Figure 3.2) by fibronectin and proceeded with lift-off and subsequent backfilling. We observed that the cell attached exclusively to the fibronectin area and thus demonstrated that this simplistic approach is also possible.

![Figure 5.8](image)

**Figure 5.8** The adsorption of RGD-modified polymer was replaced by incubation of fibronectin. Fibroblasts attached on the Fn coated 200 μm wide stripes separated by PLL-g-PEG.

One has to keep in mind that this approach presents denatured proteins to the cells, mainly due to the dipping into the NMP organic solvent. However, this result demonstrates that this denatured Fn coating still supports cell adhesion and spreading. Bathia *et al.* used this approach earlier to immobilize collagen on glass and subsequently remove the photoresist with acetone. First, hepatocytes where plated in serum-free media in order to wash away the cells on the glass background. In the second step, instead of passivating the glass, they plated fibroblasts in order to co-culture two cells types with spatially controlled populations.153
5.3 CELL PATTERN STABILITY

Although many studies have focussed on the early events of cell-surface interactions i.e., from minutes up to 24 hours, the fidelity over time of the cell pattern with respect to the underlying peptide pattern is an important issue for long term use of such cultures. By long term, we typically mean time periods between 1 day up and several weeks, which are often required for applications such as cell-based drug discovery.

Cell pattern instability can be described as cells growing out (by migration or proliferation) of the original chemical cell-adhesive patches. The consequence of such outgrowth is a loss of spatial resolution of the arrayed cell culture. Degradation of micropatterned surfaces may happen through both, cell-dependent and cell-independent processes as it has been shown previously. The following work was a collaboration with Jost Lussi and Gabor Csucs (both ETHZ) and has been submitted recently to Biomaterials.

A pattern stability study was undertaken on MAPL surfaces as well as on two other patterning techniques used at LSST, namely microcontact printing (μCP) and selective molecular assembly patterning (SMAP). These two additional techniques, briefly presented in chapter 1, also rely on PLL-g-PEG for background passivation. For this study relatively large RGD island sizes (200x200 μm² squares and dots with 200 μm diameter) were used to allow many cells to adhere on each patch. Cell outgrowth out of those patches i.e., loss of cell confinement, was taken as a characteristic measure for loss of pattern stability. By monitoring the outgrowth over time, it is possible to determine how long the different patterning methods were capable of confining cells to the original pattern geometry and compare them in terms of their performance over prolonged exposure to cell culture conditions. Note that reproducibility issues of the patterns have been
neglected and we specifically choose areas that were initially defect-free; i.e.,
where cells exclusively attached to the RGD patterns and not in the background.
The aspect of pattern quality and reproducibility is addressed in the next section,
where surface defects are discussed. Furthermore, the three patterning methods
tested cannot be compared directly for all aspects, particularly since the immobi¬
lization of ligands in the MAPL patterns is based on the grafted RGD-oligopep¬
tides as the anchoring molecule, whereas for μCP and SMAP direct adsorption of
adhesion proteins provided the required signal and physical link for cell attach¬
ment. Therefore, this study focused on the cell-passivation capability of PLL-g-
PEG applied to different patterning methods.

While cells recognized the patterns and were largely confined to the adhesive
areas created by all three techniques when evaluated at the beginning of the
experiments, pronounced loss of pattern constraints on some type of patterns
were observed after prolonged exposure to cell culture conditions. Additionally,
an unexpected effects was observed on the MAPL-niobia chips exclusively;
throughout the two weeks experiment some cells were unstably spread in the
background i.e., by simple removal of the cells from the incubator they would
retract, round up and be washed away by media change process. Potential causes
of such behavior is discussed in the next section (defects in cell patterns).

Figure 5.9 shows clear differences in the ability of the different patterns to pre¬
vent cell outgrowth. For example at day 3 (48 hours after plating) the MAPL pat¬
terns produced on TiO₂ already presented cells migrating out of the adhesive
islands, while this was not the case for MAPL patterns on Nb₂O₅. Failure of the
SMAP patterns was observed to start at day 7 and a complete cell sheet was
formed at day 13. Patterns produced by microcontact printing of proteins on
TCPS as well as the MAPL patterns on Nb₂O₅ were able to constrained cells for
at least two weeks. The increasing density of fibroblast cells on the patches over
time indicated that the cells were proliferating on these large islands. While cells in the center of the growing clusters might die due to lack of nutrient diffusion, cell proliferation was not stopped when a local confluent layer was reached in the pattern. Cells on samples prepared by μCP on TCPS as well as MAPL on Nb₂O₅ were found to grow into the medium building up tower-like cell aggregates (Figure 5.10). Such towers reached heights of up to ~150 microns and due to mechanical instability eventually broke apart upon medium exchange after 14 days in culture. Such aggregates have also been reported with C2C12 myoblasts.¹⁸⁸

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**Figure 5.9** 3T3 mouse fibroblast cells incubated on patterns created by different techniques monitored over time and imaged in DIC mode.
Chapter 5

Figure 5.10 Cells were not stopped from proliferating when confluence within the patches was reached but started to form cell aggregates that grew into the z-direction, away from the surface and into the medium. The approximate height, estimated with the focus wheel of the microscope, was about 150 μm.

Figure 5.11 depicts the relative amount of patches with significant cell outgrowth in function of culture time and pattern type. Three independent experiments were performed with each sample type. Each line is a separate experiment and the results shown are average values of at least two samples and 50 patches per sample.

Figure 5.11 Quantification of pattern stability by determining the percentage of patches that showed significant cell outgrowth. Each line represents a separate experiment where at least two samples and 50 patches were analyzed.
The fundamental difference on these patterned surfaces lies in the type of substrate material used to immobilize the PLL-g-PEG polymer. SMAP had an SiO₂ background, μCP benefits of a TCPS background while MAPL was made either on TiO₂ or Nb₂O₅. The above results do not unambiguously reveal which mechanism led to the loss of cell resistance. Cells are known to remodel their underlying ECM layer by different mechanisms. In patterning applications this activity may affect both the cell-adhesive patches and the non-adhesive background. Living cells also secrete ECM proteins and enzymes in amounts mainly depending on the culture media and the cell type. It is reasonable to hypothesize that cell-secreted proteins are deposited on top of the PEG layer without strong physical attachment to the underlying substrate but enough to support cell growth (e.g., formation of a raft-like ECM layer on top of the PEGylated surface). Also, the PEG chains could potential be oxidized under culture conditions. Another explanation could be the exchange between serum proteins and the electrostatically adsorbed PLL-g-PEG. Such a mechanism would be described as a cell-independent process.

Based on our results, two possibilities can be discarded, namely the oxidation of the polyethylene glycol chains and the idea of a protein-raft on top of the coating. These two mechanisms are unlikely since such process would be observed on all surfaces for a given time point. However, to elucidate if the pattern degradation is a cell-dependent or independent process we proposed the following experiment: the patterns were exposed to the same serum-containing medium, as used for cell experiments, and kept in the incubator at 37°C and 5% CO₂ for various time periods without cells. The medium was exchanged at the same time interval as for the patterns with cells. Then, cells were plated (10⁵ cells/cm²) on these samples (incubated in the media at different time points) and analyzed one day after seeding.
Figure 5.12 shows that the MAPL-TiO$_2$ patterns start to degrade after 1 day in media (10% FBS) + 1 day of cell culture in serum (10% FBS). Similarly, all other surfaces presented comparable pattern degradation as for the samples incubated with cells since day 1. In other words, the different types of samples degraded after different number of days as already observed in Figure 5.9. but no differences in pattern degradation times were observed if the samples were incubated with or without cells as shown Figure 5.12.

![Figure 5.12](image)

**Figure 5.12** Cell pattern stability after exposure to serum containing medium for various time periods and subsequent cells incubation for 1 day. The patterns were produced with three different techniques: Molecular Assembly Patterning by Lift-off on titania or on niobia; Selective Molecular Assembly Patterning on a silica background and microcontact printing on tissue culture polystyrene.
This demonstrates that the pattern degradation is not due to the presence of the cells but rather by the instability of the PLL-g-PEG in the presence of serum proteins. The polymer desorbed to a significant degree after 2 days in serum-containing media when adsorbed on TiO$_2$ and about 7 days on SiO$_2$. On niobia and TCPS no significant stability losses were observed. The interactions of the PLL backbone with the surfaces are occurring via multiple charges (the PLL backbone have multiple positive charges at neutral pH). The necessary negative charges are provided by the substrate. Charge densities as well as isoelectric points are known to vary depending on the material. The isoelectric point (IEP) is the pH value corresponding to a zero net charge. Any material with an IEP $<$ pH of the environment will have a higher number of negative than positive charges and thus, a negative net charge. A low IEP (relative to 7.4) may help to stabilize the adsorbed polymer. Oxides with IEP $>$ 7.4, on the other hand, were also shown to adsorb the positively charged polymer (personal communication of Janos Vörös, LSST, ETHZ). The requirement for adsorption is the presence of sufficient negative charges at the interface. However, the surface charge densities for a given pH are not readily available. Literature provides a number of zeta-potential data (potential vs. pH) on silica and titania, unfortunately no data are available for niobia. Moreover, comparative data between silica and titania are often found to be contradictory in literature. Table 5.1 presents IEP ranges believed to be reliable, based on the literature compiled by Kosmulski.$^{231}$ Also, from our experience we have observed that roughly 30% less polymer adsorbs on TiO$_2$ and SiO$_2$ compared to Nb$_2$O$_5$ (Table 5.1).
Table 5.1  Compilation of isoelectric points and PLL(20)-g[3.5]-PEG(2) adsorbed masses measured by OWLS. The masses on silica and titania are compiled masses from data internal to LSST. They were re-calculated with \( dn/dc = 0.158 \), 10% of the masses were subtracted if adsorbed in Hepes 10 mM (Hepes 1) rather than Hepes 150 mM (Hepes 2).

<table>
<thead>
<tr>
<th></th>
<th>SiO(_2)</th>
<th>TiO(_2)</th>
<th>Nb(_2)O(_5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IEP</td>
<td>&lt;3 if any</td>
<td>4.0-6.0</td>
<td>~4.4</td>
</tr>
<tr>
<td>adsorbed mass [ng/cm(^2)]</td>
<td>125</td>
<td>129</td>
<td>184</td>
</tr>
<tr>
<td>stability results</td>
<td>~7 days</td>
<td>~2 days</td>
<td>&gt;14 days</td>
</tr>
</tbody>
</table>

By comparing the IEP values, the polymer adsorbed masses and the result of the stability study it becomes obvious that the IEP does not correlate with the polymer adsorption behavior. This suggests that the affinity of the PLL on the surface is charge density dependent and not simply dependent on the IEP. Since no data are available we can only speculate about the possible origin of the observed polymer stability differences. If we hypothesize that the stability of the polymer is governed only by the amount of charges present at the interface we could assume that the negative charge density is the lowest on titania, medium for silica and highest for niobia and TCPS. Kurosch Rezwan (nonmet, ETHZ) has shown that the zetapotential of SiO\(_2\) has a higher negative value than titania suggesting a higher charge density for silica.\(^{232}\) PLL interacts with hydrophilic surfaces mostly due to electrostatic interactions. Consequently the attachment strength depends directly on the surface charge density of the substrate. If the latter is reduced, then exchange of polymer molecules with proteins becomes more likely. Note that the polymer desorption observed on titania and silica does not necessarily correlate with similar experiments performed on plasma cleaned oxide surfaces. Oxygen plasma has two important effects on the oxide surfaces; the adventitious hydrocarbon contamination layer is removed, improving the
close apposition of the polyelectrolyte and the surface, and secondly, the surface concentration of oxygen is likely to increase, which may result in a higher hydroxide surface concentration.

Our results demonstrate that PLL-g-PEG adsorbed on Nb2O5 (or TCPS) is stable for at least 14 days under cell culture conditions (10% serum containing media at 37°C, 5% CO2). Two weeks cell pattern stability, and potentially more, is considered as outstanding in the field of cell patterning.88 The most widely used system, microcontact printing of alkanethiols on gold, shows cell pattern stability up to 5-7 days.140 Pattern degradation is attributed to the oxidation of the thiolate group and subsequent desorption.105 Moreover, this system usually requires an initial incubation with cells in a serum-free medium. A few research groups have reported exceptionally long term cell pattern stability. Ma et al. were able to constrain NIH 3T3 fibroblasts for up to 28 days in 10% serum.113 Their approach was a surface-initiated atom transfer radical polymerization (SI-ATRP) of oligo(ethylene glycol) methyl metacrylate (OEGMA) monomers that were polymerized on thiolated SAMs on gold. The resulting structure is a "bottle"-brush polymer of ~20 nm thickness that presents excellent non-fouling properties mainly due to the highly hydrated OEG side chains. Griffin et al. also reported good pattern stability with an interpenetrating network of p(AAm-co-EG) resisting cell adhesion for at least 2 weeks.233 Berg et al. patterned fibroblasts on polyelectrolyte multilayers with a pattern fidelity of one months.57 Messersmith reported preservation of cell resistance up to 2 months for oxide surfaces functionalized with (DOPA)3-PEG, a surface with exceptionally high PEG chain density and an anchorage scheme derived from DOPA-containing mussel adhesive proteins.234 The common property of the above mentioned chemistries is a comparatively high film thickness (compared to typical SAMs and PLL-g-PEG)
resulting also in a high water content forcing the structure to an increased degree of swelling. Moreover, these systems are likely to be more stable both in terms of resistance to chemical changes (e.g., due to crosslinking or multivalent interactions) and adhesion strength (e.g., covalent attachment to the substrate). A high degree of hydration is believed to be an important contribution to the protein resistance, also in the PLL-g-PEG system.

Currently, a strategy is being investigated at LSST (in collaboration with Univ. of South Australia) to covalently immobilize PLL-g-PEG on a plasma-deposited aldehyde polymer film (Thomas Blättler and Mike Gabi, both LSST, ETHZ). This approach should significantly increase the stability of the polymer on all oxide surfaces, provided that the plasma-polymerized film exhibits good adhesion strength to the substrate. Another approach investigated at LSST, is the adsorption of PLL(350)-3.5-PEG(2). This higher-molecular-weight PLL has a significantly longer PLL backbone and hence, more positive charges interacting with the substrate.

To our knowledge, no publication reports any dependence of cell type in terms of difficulty in constraining cells on patterns. However, to weight out this in terms of pattern stability, we cite Andrés García: he pointed out, in a personal communication, that his lab had great difficulties in patterning MC 3T3-E1 osteoblasts on Fn patterns surrounded by oligo(ethylene glycol) (EG₃) modified alkanethiols on gold, whereas NIH 3T3 and IMR-90 fibroblasts remained constraint on the Fn islands for several days. A possible cause for these differences could potentially be related to the important quantity of ECM proteins produced by MC 3T3-E1 cells. Such difficulties can be expected from other cell types as well but are, of course, also related to the ability of the non-fouling chemistry to inhibit cell adhesion/protein adsorption. To further illustrate these differences, the example of neuronal cells on patterns is helpful; in literature, it is
often reported that laminin patterns, without any non-fouling background, is sufficient to precisely guide neurons and neurite growth on patterned 2D surfaces.

5.4 DEFECTS IN CELL PATTERNS

In the previous section the long term stability of cell patterns was discussed without considering defects located in the background and leading to pattern failure within 3-4 hours after plating. Occasionally, cells were observed to spread on the background few hours after plating and thus leading to poor pattern quality. Two different types of behavior were observed on the background, namely stable and unstable spreading. Unstable spreading refers to spontaneous cell retraction and rounding-up upon removal of the sample from the incubator. Such a phenomenon usually occurred within 1-3 min. These cells could then be washed away upon media exchange. Such behavior has also been reported with endothelial cells by Chen et al.\textsuperscript{80} Stable spreading, on the other hand, is characterized by the absence of cell shape changes upon removal of the sample from the incubator.

In order for a cell to spread, the presence of ligands (proteins or peptides) is required on the surface. For the MAPL method two possible effects may contribute to the occasional poor pattern quality: Firstly, the PLL-g-PEG coating may locally fail, resulting in adsorption of serum proteins (scratches on the edges of the samples due to tweezer handling were not considered here). The failure of the polymer to adsorb can either originate from a contaminant such as a particle that adsorbed on the surface between the lift-off and the backfilling step; a molecular contaminant in the polymer solution or photoresist residues. However, all three possibilities are highly unlikely since on control MAPL chips, where PLL-g-PEG was adsorbed in both steps (a homogeneously coated MAPL surface), cells were never found to spread. The control experiments were generally carried out at the
start of a new pattern production campaign. They turned out to be very useful since they allowed us to rule out potential quality problems with the substrate, the lift-off quality or the polymer. The second possible cause of pattern defects is the presence of RGD-modified polymer in the background. Upon adsorption of functionalized polymer (stage II, Figure 3.2), the PR is also coated by the RGD-polymer. We reported in Section 4.1.2.3 that PLL-g-PEG also adsorbs onto the oxide surface when dissolved in NMP. Functionalized polymer can potentially adsorb on the background either, during the NMP rinsing step or in the sonicated NMP bath, provided that polymer molecules are in the solution. We recall that the NMP rinsing step has precisely the task to prevent the presence of polymer molecules in the NMP bath. This was done by partially removing the PR and its bound polymers by flushing the surface with NMP (Figure 5.13). During the NMP rinsing step one can optically observe the dissolution of the reddish colored PR, meaning that the bare oxide is exposed within 2-3 seconds after the first exposure to NMP. Therefore, there is a risk that the functionalized polymer re-adsorbs from the thin remaining film of NMP. Interestingly, when defects were present on the surface, they were usually located near the bottom edge of the sample with respect to the NMP flow (Figure 5.14). This observation is likely related to the high concentration of functionalized molecules in the NMP "drop" formed at the lower edge of the sample. These molecules can therefore rapidly re-adsorb on the surface resulting in a number of integrin binding sites outside the adhesive patches.
Figure 5.13 Schematic of the NMP rinsing step. Note that the NMP solvent flow (from a squeeze bottle) is directed to the upper part of the sample accompanied by a lateral movement for 3-5 s. The sample is held with a slight negative angle (facing downwards).

Figure 5.14 Bottom part of sample composed of 60x60 μm². Defects are characterized by the spreading of cells between the adhesive islands.

Massia and Hubbell reported that 1 fmol/cm² of RGD peptide (RGD ligands separated on average by 440 nm or 5.9 RGD/μm²) was enough to induce maxi-
mal spreading of human foreskin fibroblasts (4h in serum-free media). Focal complexes and cytoskeleton organization (actin microfilaments) required only 10 fmol/cm² of the same peptide (RGD ligand separated on average by 140 nm or 59 RGD/µm²). The RGD surface densities used in this work were 4.8 pmol/cm² and thus a coating of 1 fmol/cm² represents only 0.02% of a PLL-g-PEG/PEG-RGD monolayer. Based on a hexagonal closed packed lattice (Equation 5.1; \( n_{RGD} \) is the peptide density), 4.8 pmol/cm² (=28’000 RGD/µm²) correspond to an average peptide separation of 6.5 nm.

\[
L = \sqrt[3]{\frac{2}{\sqrt{3} \cdot n_{RGD}}}
\]

Equation 5.1

This illustrates that a few RGD molecules adsorbed on the surface may be sufficient to induce cell spreading and attachment. Therefore, this could explain the instable cell spreading observed occasionally when a small perturbation (e.g., temperature difference between lab and incubator) induced cell retraction. In the cases where cells were found to be stably spread, could thus indicate the presence of a higher amount of PLL-g-PEG/PEG-RGD in the background. More recently Spatz and coworker have published that formation of focal adhesion was greatly enhanced, in four different cell types, when the average inter-peptide spacing was reduced from 73 nm (220 RGD/µm²) to 56 nm (370 RGD/µm²). This results provides additional proof that very low densities of RGD ligands are required for cell spreading. Note that only formation of FA and actin network were investigated and that these minimum ligand densities may not be sufficient for triggering specific intra-cellular pathways.
5.5 Cell adhesion strength

During the development of the MAPL chips we observed that careful handling was necessary in order to prevent detachment of the cells from the patterns while exchanging the media. Although the cells did spread and form focal adhesions on the RGD modified polymer it was common to lose a significant number of cells during vacuum aspiration of the media. We also observed, that under similar aspiration conditions, less cells were detached when cultured on TCPS. Therefore, it is advised to manually pipette out the media rather than using a strong vacuum suction. Figure 5.15 shows an example of two fixed cells, where one was partially removed from the surface and became distorted.

![Figure 5.15 MG63 cells on 60x60 µm² pattern stained for F-actin. One cell was spread on the squared island, while the other partially detached from the surface and became distorted.](image)

To quantify, this apparently weaker adhesion strength on RGD compared to ECM or Fn coated surfaces, we used a spinning device. In brief, round glass cover slips (diam. 25 mm) were coated either with human plasma fibronectin or with PLL-g-PEG/PEG-RGD. Since the experiment was performed on silica and
not on niobia the ligand surface density was estimated based on results of Table 5.1. Approximately 32% less polymer is adsorbed on silica compared to niobia, which results in an RGD density of 19’000 µm² for a pure PLL-g-PEG/PEG-RGD coating. A second set of samples was coated with a mixed solution (mixed 1:3 with PLL-g-PEG) to obtain 4’800 µm². In terms of average inter-peptide distances they corresponded to 8 nm and 16 nm, respectively.

NIH 3T3 fibroblasts were seeded (~100’000 cells per disk) and incubated for 16 hours in 10% NCS added media. The spinning device was equipped with an electric motor allowing RPM adjustment. Each disk was spun in a separate run. The disk was vacuum-glued on a horizontal support and placed in a spinning chamber filled with PBS, 2 mM dextrose and 5% dextran. The rotation speed was continuously increased (within 30s) up to 2000 rpm for the RGD coated disks and 3500 rpm for the Fn coated disks. The cells were spun for 5 min each. After removing the disk, cells were fixed in 4% ice cold formaldehyde for 5 minutes follow by a 3 minutes rinse of 1% Triton X-100. The cell nuclei were stained with ethidium homodimer for 30 min at 37°C followed by a rinse with PBS. A motorized stage microscope was used to count the number of cells (stained nuclei) with respect to the radial position (61 fields recorded). The number of cells of each analyzed field was normalized to the number of cells present in the central field of the disk. The shear stress, \( \tau \) [dyn/cm²] along the disk radius \( r \) [cm] can be expressed by Equation 5.2, where \( \rho \) [g/cm³] and \( \mu \) [dyn s/cm²] are the fluid density and viscosity, respectively and \( \omega \) [s⁻¹] the rotational speed.

\[
\tau = 0.8r\sqrt[3]{\rho \mu \omega}
\]

Equation 5.2

The data were fitted with a sigmoidal curve in order to read out the mean adhe-
sion strength corresponding to the shear stress where 50% of the cells detached from the surface. Figure 5.16 are two representative shear force vs. cell fraction curves taken with the two tested surface chemistries. The mean adhesion strength ($\tau_{50}$) for the tested surfaces are reproduced in Figure 5.17. Note that 10 dyn/cm$^2$ correspond to 1 N/m$^2$. It is found that the adhesion strength of NIH 3T3 fibroblasts on the RGD surfaces is approximately half of that found for Fn coated surfaces. The adhesion strength measured on the two different RGD surface densities were found to be similar; by decreasing the density from 19,000 ligands/\(\mu\)m$^2$ to 4,800 ligands/\(\mu\)m$^2$ a decrease of only 10% in $\tau_{50}$ was measured. This slight difference suggests that, at these RGD surface concentrations, the differences in cell response, in term of adhesion strength, are rather negligible.

Figure 5.16 Adhesion strength of murine NIH 3T3 fibroblasts represented by the cell fraction (normalized to the number of cells in the center of the sample) vs. shear stress. a) Fn coated glass and b) PLL-g-PEG/PEG-RGD (4’800 RGD/\(\mu\)m$^2$) coated glass.
Figure 5.17  Mean adhesion strength of murine NIH 3T3 fibroblasts on glass coated with Fn or with PLL-g-PEG/PEG-RGD with RGD peptide densities of 19’000 and 4’800 RGD/μm².

For comparison, a coating of human plasma fibronectin on methyl-terminated alkanethiols on gold showed values of 200±30 ng/cm². Based on a molecular weight of 440 kDa for Fn, the surface coverage is 0.45±0.07 pmol/cm² or 2’800 Fn/μm² i.e., a significantly lower amount of ligands compared to the synthetic RGD surfaces. Based on the publications of Massia et al. and Arnold et al., discussed in the previous section, the number of ligands required for cell attachment (FA and stress fibers formation) is in excess by two orders of magnitude on our RGD surfaces. Therefore, the reduced adhesion strength cannot be attributed to an insufficient number of RGD-anchoring points. The extensive research focusing on fibronectin at surfaces provides a number of clues to explain the differences observed between Fn and RGD. Andrés Garcia and collaborators have demonstrated that the PHSRN synergy site localized in the ninth type III domain of Fn is critical for binding of the α5β1 integrins. It was also
shown that the presence of only one domain (RGD or PHSRN) contributes little to cell binding strength, but the combination acts synergistically resulting in a tenfold increase of the adhesion strength. The $\alpha_v\beta_3$ integrins bind only to the RGD domain and thus, the absence of synergy site results in poor mechanical coupling. This suggests that the use of the RGD synthetic peptide alone does not provide robust mechanical coupling to the activated receptors. Also, cells attached to a fibronectin coating actively remodel their substrate. Viola Vogel and coworkers identified, via intramolecular FRET, that Fn assembled in fibrils, is stretched by mechanical tension exerted by the cells. This molecular unfolding is likely to induce functional changes such as exposure of cryptic sites and modification of distances between the different epitopes. It is hypothesized that mechanical tension could serve as an additional mean by which cells alter the biochemical signals presented by their environment. These cell microenvironment modifications are potentially also influencing directly the cell adhesion strength. Rajagopalan et al. measured over two times higher cell-contractile forces on Fn compared to an RGD surface with identical ligand density. However, both surfaces (same molecular density) induced differences in spreading area; higher on Fn than on RGD. Here, the use of micropatterns to control the spreading would easily clarify if the differences in contractile forces are, in this regime, spreading dependent.
6.1 THE POTENTIAL OF VESICLE ARRAYS

The combination of lipidic vesicles and chemical microarrays is a promising novel platforms for the immobilization of transmembrane proteins or nano chemical reactors. The inert and tight environment of the lipidic wall is specially well suited for the study of molecular recognition events of a few (or single) molecules. Besides of their use as nanoreactors for volumes as small as zeptoliters, the lipid bilayer ideally mimics the membrane of cells. Native transmembrane proteins such as channel proteins are known to denature easily when deprived of their natural environment. Studying the activity of such proteins in living cells is still highly challenging and therefore novel routes are investigated. Vesicles (synthetic or native) that carry such proteins within their membrane are expected to have substantial potential for applications in diagnostics and drug screening, in view of the fact that membrane proteins make up a third of the human proteome; also, more than 50% of the currently used drugs act on membrane proteins. However, a challenging task remains: the specific immobilization of these vesicles on surfaces as an array of individually (or small clusters of) addressable entities, while preserving their native functionality.
In certain applications an ordered array may not be required and the random attachment of vesicles on to the surface may be sufficient as shown by Michel et al. with the nanoSMAP technique.\textsuperscript{155} The group of Horst Vogel pioneered the fabrication of arrays of few vesicles with micro contact printing.\textsuperscript{243} Höök and coworkers created prepatterns of gold on SiO$_2$ and subsequently modified the Au with single stranded DNA fragments, while the SiO$_2$ background was modified with a lipid bilayer rendering it inert.\textsuperscript{244} Upon incubation in a suspension of DNA-tagged vesicle, specific DNA hybridization was observed and consequently local immobilization of liposomes was achieved. This strategy was used in our work as a result of a fruitful collaboration between B. Städler (diploma student, co-supervised by J. Vörös and myself) and the laboratory of F. Höök in Chalmers Univ. Sweden. The goal was to form arrays of vesicles on a MAPL platform while exploiting the specificity provided by the DNA recognition system. This work was recently published in Langmuir.\textsuperscript{207}

### 6.2 IMMOBILIZING VESICLES ON DNA MAPL CHIPS

Immobilizing DNA-tagged vesicles in a desired pattern was performed by creating arrays of single-stranded oligo-nucleotides. We applied the MAPL process to create such a DNA-chip. The surface modification was carried out in the following manner: first, a PLL-g-PEG/PEG-biotin array with PLL-g-PEG background was created on a glass chip (coated with Nb$_2$O$_5$) by using the MAPL process. Complexes of streptavidin (or neutravidin) and biotinylated-DNA were prepared in solution prior to adsorption. The immobilization of biotin-SA complexes has been demonstrated to be much more efficient and controlled than the standard step-by-step protocol.\textsuperscript{245} Upon incubation, the biotin spots were converted into an array of multiple ss-DNAs (ss: single strands composed of 20
Vesicles were tagged with a complementary DNA strand (cDNA) via a cholesterol group prior to hybridization on the MAPL surface (Figure 6.1).

Figure 6.1 Schematics of the multistep surface modification process for the immobilization of intact vesicles using DNA as coupling agent.

Since liposomes have to be kept in an aqueous media at all times (to prevent their disruption), all adsorption steps were carried out in a "home-made" flow chamber placed on the microscope stage. The adsorption of the streptavidin-DNA complexes as well as the vesicle immobilization was thoroughly investigated and optimized with OWLS and QCM-D by B. Staedler and the results published recently.\textsuperscript{207}

Figure 6.2 shows the CLSM images of a) labeled streptavidin and b) labeled vesicles. In both cases an excellent contrast was found (no non-specific signal was detected). These results show that the MAPL chips allow the easy production of homogeneous DNA arrays as well as vesicle arrays. Furthermore, the ability to control the amount of biotin in the patches paves the way to the production of patterned surfaces with a controlled number of vesicles per island.
Figure 6.2  a) CLSM image of the adsorption of biotinDNA-streptavidin Alexa Fluoro633 complexes onto 60x60\(\mu\)m\(^2\) spots of PLL-g-PEG/PEG-biotin. b) The complexes of cholesterol cDNA tagged and NBD-labeled vesicles hybridized selectively onto the underlying patterns of biotinDNA. The PLL-g-PEG covered background was resistant to both streptavidin-DNA and vesicle-DNA adsorption. Two different filter sets were used to acquire the images and no cross-talk between the fluorophores was detected.

Heterogeneous arrays of surface-immobilized nanocontainers have a potential for performing high-throughput experiments for research applications as well as drug discovery.\(^{240}\) The above presented approach allows the production of highly homogeneous and reproducible vesicles patterns. However, patterns of the same type of vesicles do not fulfill the criterion of a high-throughput platform. The ultimate goal is to produce heterogeneous vesicle patterns in the micron range, for multiple vesicular arrays (chapter 7), or in a nanoarray format, for the immobilization of single vesicles (chapter 8). The DNA binding strategy is well suited for this task since oligo-nucleotide hybridization is highly specific; availability
and type of sequences are virtually infinite. The bottle-neck to this approach is
the production of a chip composed of different DNAs for each spot. To explore
the feasibility of such a surface, while keeping the advantage of the MAPL chip,
we spotted nanoliters of streptavidin on a MAPL surface composed of biotin
patches. With this, we explored the feasibility of spotting different SA-DNA
complexes on different biotin spots. In chapter 7, first results, performed on a
piezo-spotting machine, are presented as well as a discussion on the potential use
of MAPL surfaces for better defined protein microarrays.
7.1 INTRODUCTION INTO PROTEIN MICROARRAYS

As an immediate consequence of the large-scale genomic sequencing efforts, a strong interest has emerged in analyzing the function of the DNA-encoded information on a similarly global scale. Although DNA/RNA arrays give information on the genetic defects that may cause diseases, protein microarrays provide precious information about corresponding functional states. Many aspects of modulation and regulation of cellular activity cannot be investigated on the level of nucleic acids, but require an analysis of the proteome. For example, several studies in yeast and higher eukaryots demonstrated a poor correlation of mRNA and protein level.\textsuperscript{12} Also, the true targets of medicines are proteins and not genes.\textsuperscript{13,246} A major effort is currently made in producing protein, peptide, antibody and polysaccharide arrays analogous to the DNA chips.\textsuperscript{9,247,248} However, the difficulties to produce protein arrays that work reproducibly, quantitatively and efficiently, are as great as the promises and expectations of such analytical tools.\textsuperscript{11,249} Before such novel multiplexed sensing platforms become a standard tool in biosciences labs, new strategies and ideas have to elevate the field from its current state to a reliable "off-the-shelf" device. One major complication is the
inherent poor stability of proteins as opposed to nucleotide fragments. Most of the proteins are highly sensitive to their environment and are likely to lose their native functionality (secondary and tertiary structure) once they are out of their natural milieu. Recently, Lee and Mrksich reviewed the technical challenges inherent to the production of protein microarrays for quantitative assays. Besides of the importance of preventing non-specific adsorption, with better strategies than the commonly used BSA (bovine serum albumin) blocking scheme, they strongly emphasized the importance of being able to precisely control the orientation and the density of surface-immobilized ligands. For example, the commonly used hydrogels (3D architecture) do not provide control over such features.

Nowadays, the "standard" approach to produce high density arrays of proteins is to spot nanoliters of the molecules of interest onto a homogeneous epoxy activated glass slide. Two main spotter technologies are currently available; the contact spotting, where the compounds are deposited by the physical contact of a pin with the surface, and the non-contact method where a piezo-crystal jets the molecules onto the surface. Depending on the surface chemistry and the printing buffer conditions, spots in the range of 10-250 µm in diameter are produced. Also, for a given binding chemistry (e.g., epoxy or amines), the spot shape was observed to vary considerably depending on the type of proteins spotted. This problem is significant since the essence of protein microarrays is to generate patterns of hundreds to thousands of different proteins on a given surface chemistry. Upon binding reaction (usually overnight) the non-spotted surfaces are usually passivated with BSA to prevent the excess of proteins to adsorb on the background during the washing step. One limitation inherent to this popular approach is that the BSA passivation has a relatively low efficiency in preventing the adsorption of many different proteins. Moreover, when small proteins are
spotted they are often poorly accessible due to the surrounding BSA molecules.247

In this context, we proposed to combine the MAPL surfaces and the spotting techniques with two aims: i) to improve the spot homogeneity and shape by preventing the excess of proteins from smearing onto the background during the washing step and ii) test the feasibility to produce heterogeneous streptavidin-DNA arrays to pattern and sort differently DNA-tagged vesicles. This preliminary study was done in collaboration with Dr. Jens Sobek from the Functional Genomic Center Zürich (FGCZ is a joint institute of the University of Zürich and ETHZ, located on Irchel campus).

7.2 Spotting streptavidin on MAPL chips

We prepared MAPL surfaces with biotin patterns of 100x100 µm² and a center-to-center distance of 400 µm (see chapter 10 for details) as well as homogeneous surfaces of pure PLL-g-PEG/PEG-biotin and pure PLL-g-PEG. The substrates were low auto-fluorescent microarray slides (Schott) that we sputter-coated with 12 nm transparent niobium oxide. Each slide was carefully aligned (clean room) so that the structures were parallel to the slide edges. Prior to the MAPL surface modification, the coordinates of the first spot, were measured under a light microscope. This step was necessary since no reference point is visible on the fully processed sample (completely transparent).

We first spotted (Perkin Elmer piezo-arrayer at FGCZ, Irchel) alexa-546 labeled streptavidin (SA) solutions on slides homogeneously coated with PLL-g-PEG/PEG-biotin (polymer FD10) of 26.6 pmol/cm² (biotin). Printing buffers were either 10% glycerol or 0.5% trehalose (both in PBS 1x), two commonly used buffer in protein printing.252 Also, four different SA concentrations were
tested (0.3, 1, 3, 10 μM). The spot sizes were varied by printing either 1, 2, 3 or 4 drops per location. Each drop corresponded to a volume of approximately 250 pL. The slides were scanned with a GSI lumonics confocal scanner before and after washing. Photomultiplier (PMT) and laser power were adjusted for each slide dependently on the fluorescent signal.

Figure 7.1a shows a slide composed of two separate arrays of spotted SA before washing (PMT 30%; laser 70%). The left array was printed using a 10% glycerol buffer, while the array on the right was printed with 0.5% trehalose. For both buffers we observed an increase in fluorescent intensity when SA concentration was increased. However, for a given concentration, the spots printed with glycerol systematically displayed higher intensities than the spots printed with trehalose. Upon washing (Figure 7.1b) we observed extensive smearing due to the excess of SA (PMT 50%; laser 70%). This illustrate the fast binding kinetic between biotin and SA. The washing step was carried out by manually pouring 200 ml of PBST (PBS + 0.15% tween-20 surfactant) directly on the slide, for approximately 10 s, and then immediately holding the slide for 30 s under flowing DI-water. The slides were dried in a centrifuge at 500 rpm for 5 min.
Protein microarrays

Figure 7.1 Alexa-546 labeled streptavidin spotted on a homogeneously biotinylated surface (26.6 pmol/cm²) of PLL-g-PEG/PEG-biotin. a. before washing, b. after washing, c. slide printed with various number of drops in order to investigate the spot size (after washing).

The spot homogeneity was significantly better when dispensed in presence of glycerol rather than trehalose, implying that glycerol is a better solvent for printing SA on such slides. The best spot homogeneity was achieved at the highest SA concentration, namely 10 µM. Figure 7.2 is the intensity profile corresponding to the dashed line in Figure 7.1b. The signal is nicely square-shaped and no "donut-effect" was visible (higher protein concentration on the outer edge compared to the spot center).
Chapter 7

Figure 7.2  Intensity profile of the dashed line in Figure 7.1b. Spots were printed with a 10% glycerol, 10 μM SA and a volume per spot of 1 nL.

The spot size was investigated by printing various volumes (1, 2, 3 or 4 drops/spot) within the same slide and with the same spotting configuration (Figure 7.1c; PMT 70%; laser 70%). We observed that the spot diameter was mainly dictated by the number of drops and, to a smaller extent, by the SA concentration as shown in Figure 7.3. Spots printed at 0.3 μM were not detected under these scanning parameters and were thus not considered.

Figure 7.3  Spot diameter as a function of the printed volume and the SA concentration.

We hypothesized that we could potentially prevent the commonly observed smearing by rendering the non-spotted areas resistant to the adsorption of SA. The idea was to spot SA on PLL-g-PEG/PEG-biotin functionalized MAPL pat-
terns (with PLL-g-PEG in the background). To test this concept, we first spotted SA on MAPL patterns composed exclusively of non-biotinylated PLL-g-PEG to determine the resistance of the PEGylated surface to non-specific adsorption of SA. Figure 7.4a is the scanned slide before washing (PMT 5%; laser 70%). Upon washing (Figure 7.4b) all spots printed with glycerol completely disappeared. In view of the extreme sensitivity of the scanner (detection limit given by manufacturer: 0.1 fluorophore/μm² or < 800 fluorophores/spot of 100 μm diam.) and PMT/laser set to their maximum values (both 90%) this surface is essentially free of SA. It is worth noting that this slide was stored for 12 days in a 50 ml test tube in air at room temperature before spotting. Another analogous slide was spotted 10 months after preparation and did not display significant non-specific signal (data not shown). These results also demonstrate that the washing process was adequate, provided that the compounds were printed in 10% glycerol. The spots printed in 0.5% trehalose could not be washed away, suggesting that the spots dried at the surface. In this case, less than 10 min elapsed between the end of the printing and the washing step and thus, demonstrated that the trehalose-containing buffer cannot be used for such applications under the chosen conditions.
We conclude that PLL-g-PEG inhibits the non-specific adsorption of SA spotted in glycerol-based solution provided that no protein drying occurs at the surface. We observed that some drying took place (ring shape) when spots, printed in presence of 10% glycerol, where allowed to dry for 3 hours in uncontrolled atmosphere (data not shown).

In a next step we spotted SA on a MAPL chips. We produced squared biotin patterns of 100x100 μm² to better document the effect of the MAPL pattern on the spot geometry. SA in four different concentrations and two different buffers were spotted with a volume of 4 drops each. The volume was set so that the spot diameter would exceed the diagonal of the biotin pattern. Prior to the MAPL process, the coordinates of the first spot were measured from the photoresist pattern with respect to the upper left corner (when the slide is hold vertically). The fully processed MAPL samples were then placed in the spotter and the origin set on
the upper left corner via the camera (spatial resolution of ~5 μm).

Figure 7.5 shows a series of spots before and after washing. Two observations were made upon washing; first, the printed spots did not "land" exactly on the underlying biotin squares and secondly, fluorescent signal was detected in the background (between the spots). The lack of alignment was attributed to a mismatch between the sample and the spotter orthogonal reference systems. (This spotter-related problem has been solved since then). It was more surprising that fluorescence signal was measured in the non-biotinylated areas (outside the squares) since this was not observed earlier (with the 1x1cm² dipped into SA solution, see chapter 4). Based on the negative control experiment (MAPL samples with pure PLL-g-PEG coatings), the only reasonable explanation for this observation is the presence of PLL-g-PEG/PEG-biotin in the background. Such defects have already been identified with PLL-g-PEG/PEG-RGD in Section 5.4.

Figure 7.5  Printed MAPL slide a. before washing (PMT 30%; laser 70%) b. after washing
(PMT 70%; laser 70%) and c. after washing (PMT 80%; laser 90%). Note that SA was only spotted within the dashed square area.

To prevent this in future we propose a slight modification in the MAPL process in order to circumvent this problem by reducing the photoresist (PR) lift-off speed and thus, prevent the rapid re-adsorption of the PLL-g-PEG/PEG-biotin. Instead of rinsing the surface with NMP, which is highly uncontrolled on such large slides, we would dip the MAPL sample into a mixed solution of NMP and water, in order to dissolve only a thin homogeneous layer of photoresist. By this we would solubilized the PR-adsorbed PLL-g-PEG/PEG-biotin without giving it the opportunity to re-adsorb on the oxide background. In a next step the slide would be dipped into a pure NMP-containing beaker for complete PR removal. The optimization of the parameters for this step will be investigated in a future development.

In this preliminary study we determined: the spot size, in function of the printed volume and concentration; the adequate buffer (10% glycerol in PBS 1x) and the proper slide washing protocol. These basic parameters will be useful for future experiments.

As a further proof of concept we deposited a large drop (40 µL with a micro-pipette) of 0.3 µM SA solution (10% glycerol) on a patterned microarray. We chose this low concentration in order to minimize the background signal present after washing. The dynamic range of the scanner was set (PMT and laser) so that the highest intensity was just below saturation (16 bit information depth is $2^{16} = 65’535$ intensity levels [a.u.]). Figure 7.6a was acquired with 70% PMT and 70% laser power. The average signal to background ratio was approximately 100. However, the smearing below the squares revealed that the background signal was not homogeneous (due to the NMP rinsing step).
Figure 7.6  A 0.3 μM SA solution was incubated over a biotin functionalized MAPL pattern (100x100 μm² squares). a. selected region obtained by scanning with 70% PMT and 70% laser. b. surface plot of the fluorescent intensities. c. intensity line profile corresponding to the dashed line of figure a.

The mean intensity and standard deviation were determined for each square. The average of the means of 36 spots was to be 56’400 and the mean of the 36 standard deviations 3’900. If the data of all 36 spots are merged into a single square, the mean intensity is 56’400 with 7% standard deviation. These highly promising results confirm the findings in Figure 7.6 b. and c., namely sharp edges and rather "flat" intensity planes. The consistency from square to square is given by the standard deviation calculated with the average intensities of the 36 spots. This value was found to be 2’300 or 4% variation.
These results demonstrate both the excellent homogeneity of the biotin distribution within the patterns but also across the microarray chip thus, providing a realistic basis for the future development of improved spot quality thanks to designed surface patterns.

In terms of immobilization schemes, an alternative to the standard biotin-SA coupling\cite{208} is the NTA-Ni-histag complexation (see Section 7.3).

### 7.3 PLL-g-PEG/PEG-NTA AS A DOCKING PLATFORM

Nitrilotriacetic acid (NTA) - histidine interactions is routinely used for measuring binding constants and purification of proteins.\cite{253} The high binding specificity of hexa histidine tagged biomolecules with NTA-chelated Ni\textsuperscript{2+} ions was recently exploited in commercially available sensor chips (e.g., Biacore, Qiagen). Rigler et al. were able to immobilize membrane proteins embedded in 2D lipid bilayer.\cite{119} The lipid bilayer was functionalized with 6xhistidines and stabilized by the underlying NTA-SAMs. The NTA-6xhis interaction is known to be reversible.\cite{253,254} The dissociation constant of the histidine-NTA system is relatively high (K\textsubscript{D} 10\textsuperscript{-6} M) in comparison to biotin-streptavidin (10\textsuperscript{-15} M).\cite{255-257} The histag can be coupled to the N- or C-terminus of a protein, making it possible to control the orientation of the biomolecules at the surface, an important aspect for efficient antibody-protein recognition.\cite{258} Also, the libraries of recombinant antibodies tagged with 6xhistidines is constantly growing.

We produced a MAPL array with PLL-g-PEG/PEG-NTA patterns (60x60 \(\mu\text{m}^2\)) surrounded by non-functionalized PLL-g-PEG (Figure 7.7). The NTA-functionalized polymer was synthesized by Gouliang Zhen (LSST, ETHZ).
The patterned samples were exposed to an aqueous NiCl$_2$ solution followed by incubation with GFP-6xhistag (green fluorescent protein with 6xhis on the C-terminus). The fluorescence images in Figure 7.8a demonstrate the specific coupling of the GFP-histag to the NTA functionalized patches. Upon addition of imidazole (competitive binding with histidines), the proteins detached and were washed away (image b). To demonstrate the reversibility of the binding, GFP-histag were re-injected (image c) and washed with buffer (image d).

Figure 7.8  CLSM images; a. coupling of GFP-histag proteins on NTA functionalized MAPL chips. b. desorption of the protein upon addition of imidazole. c. reloading of GFP-histag. d. buffer rinse.
These preliminary experiments demonstrate that NTA-functionalized PLL-g-PEG is compatible with the MAPL process and reversible. We believe that the combination of NTA chemistry, MAPL surfaces (controlled NTA density and high interaction specificity) and spotting technology is a promising approach towards future protein/antibody microarrays characterized by excellent spot definition, improved presentation of capture molecules and reversibility for multiple chip use.
Nanopatterning with MAPL

8.1 Chemical nanopatterns for applications in biosciences

The ability to immobilize proteins on sub-micro- to nanometric sized areas has become a major challenge for the development of bioengineered surfaces. The ongoing technological advances are partially driven by the aim of broadening the understanding of a variety of surface-mediated biological recognition events. Micropatterned surfaces are known to influence cell function through surface-triggered interactions and controlled spreading (chapter 1). However, little is known on how spatially controlled arrays of single (or few) ligands affect living cells. Such biochemically nanopatterned surfaces are believed to provide a better insight into integrin clustering and ultimately integrin-mediated intracellular pathways. Also, the ability to spatially control the immobilization of a low number of molecules, down to single protein or DNA molecules, will provide better platforms for the study of single molecular events as for example with AFM force measurements. Also, better sensitivities are expected from such nanoarrays. As an examples, regular nanopatterns, produced by dip pen nanolithography, of antibodies against HIV, showed a 1000x increase in sen-
sitivity compared to conventional enzyme-linked immunosorbent assay (ELISA). Regular arrays of predefined spots greatly facilitate such experiments because the coordinates of each spot is known and thus enables rapid access to the locations of interest. This will greatly increase the amount of data gathered in a given time and also give a more realistic sense to automation in nanotechnology. Short access time may also be crucial in experiments involving unstable compounds. Production of arrays of single nanoreactors such as micelles or vesicles also depends on the availability of chemical nanopatterns. Loaded vesicles containing femtoliters of reagents arranged in an array format constitute a promising surface platform for combinatorial chemistry. Immobilized vesicles are also considered to have the built-in potential to act as a protein carriers for transmembrane or water-soluble proteins. Chemical nanopatterns of continuous tracks of motor-proteins (e.g., kinesin) are also envisioned as an active nano-cargo transport systems (e.g., by "decorating" microtubules with the compounds to be shipped) as has already been demonstrated on micropatterns.

Several patterning strategies have been developed to produce biologically relevant patterns at the nanometer scale and can be found in several excellent review papers. The nanopatterning processes are conveniently categorized either as serial or parallel. The most commonly used serial methods are electron-beam writing, focused ion beam and scanning probe based lithography. Their main disadvantages are high cost and long writing time (a number of writing techniques such as dip-pen lithography using multiple inkers in parallel are, however, more efficient). On the other hand, they are advantageous in terms of their flexibility in generating any types of pattern shapes, from highly periodic patterns to heteroclite motives. Examples of parallel techniques producing more or less random pattern distributions are polymer de-
nanopatterning with MAPL

testing and colloidal lithography. The most widely accepted parallel techniques for producing sub-micrometer patterns in a regular manner are micro-contact printing, organized colloidal lithography, X-ray interference lithography and nanoimprint lithography (NIL).

Independent of the technique used to create the biomolecular nanopatterns, the critical requirement remains the ability to avoid non-specific binding of proteins or biomolecules in general (Section 1.4). The uncontrolled presence of proteins on an array will most likely affect the outcome of the assay and thus bias the results. Therefore, the non-interactive areas of the pattern require special attention, through designed surface chemistry, in order to ensure a very low background signal.

Our approach to achieve 2D regular chemical nanopatterns was to combine nanoimprint lithography with the MAPL surface modification process. The nanoimprinting was performed in close collaboration with the Paul Scherrer Institute (PSI) in Villigen, Switzerland and with Daniela Pasqui, a visiting PhD candidate from University of Siena, Italy. The results were published recently in Nanoletters.

8.2 Combining Nanoimprinting and MAPL

Schematics of the NIL process are presented in Figure 8.1; silicon wafers (Wafernet) and Pyrex plates (SensorPrep) (0.5 mm thick) sputter-coated with 12 nm transparent niobium oxide (Nb$_2$O$_3$) were used as substrates. These substrates were then spin-coated with a thin film of 125 nm of poly(methyl methacrylate) (PMMA, molecular weight = 25 kg/mol), followed by a soft baking for 1 min at 170 °C. When micrometer featured stamps were used, the spin-coated PMMA layer was 330 nm. For NIL, various stamps containing either micro- or nanopat-
terns of lines, dots, and stars were fabricated using a Leica Lion LV1 electron-beam writer with subsequent reactive ion etching (RIE) of silicon. The stamps were then coated with an anti-adhesive layer and then replicated by imprinting into the PMMA-coated substrates at a temperature of 180 °C and pressure of 50 bars. The size of both stamps and substrates was $2 \times 2 \text{ cm}^2$ with the nanostructures located in the central $1 \times 1 \text{ cm}^2$ of the stamp area. The PMMA layer that remained on the bottom of the imprinted structure (Figure 8.1, stage III) was subsequently removed by O$_2$ RIE operated at a flow of 21 sccm, a chamber pressure of 20 mTorr and an RF power of 21 W. This resulted in the creation of a nanostructured surface with PMMA/Nb$_2$O$_5$ contrast (Figure 8.1, stage IV). Note that the PMMA is rendered negatively charged and more hydrophilic during the O$_2$ dry etching process as demonstrated by Chai et al. The PMMA/Nb$_2$O$_5$ contrast produced by NIL was then converted into a biologically relevant pattern using the MAPL process as described earlier. Note that the protocol for "nanoMAPL" slightly differs from the "microMAPL" since the lift-off had to be optimized for PMMA rather than for the photolithography resist. The detailed protocol can be found in chapter 10.
Figure 8.1 The NIL process: I. Spin coating of PMMA and pre-baking, II. Imprinting the PMMA layer using the silicon stamp, III. Demolding of the stamp from the substrata, IV. Anisotropic O₂ RIE for the “window opening” down to the niobium oxide. From this point the MAPL process was applied: V. The prepatterned sample is dipped into an aqueous solution of PLL-g-PEG/PEG-biotin, VI. Lift-off of the PMMA in acetone, VII. The background is filled with non-functionalized PLL-g-PEG, VIII. Streptavidin specifically bound to the biotinylated areas.

8.3 CHARACTERIZATION AND OPTIMIZATION OF THE NANOPATTERNING PROCESS

8.3.1 Quality of the PMMA lift-off on homogeneous surfaces

XPS results

The spin-coated PMMA film was found more difficult to remove from the niobia surface compared to the S1818 photoresist used for micropatterning via photolithography. For this reason, different lift-off conditions were tested. Two PMMA stripers were compared, namely acetone and NMP. Lift-off (LO) was conducted at room temperature (RT) but also in an heated ultrasonic bath (40 and 50°C). Different sonication times were applied (5 and 30 min). Table 8.1 lists the
elemental composition after lift-off and the corresponding parameters.

Table 8.1 Atomic percentages measured by XPS. Various lift-off conditions were tested for complete PMMA removal. Peak areas of C1s, O1s and Nb3d were used as a basis for quantification.

<table>
<thead>
<tr>
<th>lift-off (LO) conditions</th>
<th>% C</th>
<th>% O</th>
<th>% Nb</th>
</tr>
</thead>
<tbody>
<tr>
<td>PMMA (no LO)</td>
<td>78</td>
<td>22</td>
<td>0</td>
</tr>
<tr>
<td>NMP, 5min, RT</td>
<td>40</td>
<td>45</td>
<td>15</td>
</tr>
<tr>
<td>NMP, 30min, RT</td>
<td>21</td>
<td>55</td>
<td>24</td>
</tr>
<tr>
<td>NMP, 30min, 50°C</td>
<td>18</td>
<td>58</td>
<td>24</td>
</tr>
<tr>
<td>acetone, 5min, RT</td>
<td>34</td>
<td>48</td>
<td>18</td>
</tr>
<tr>
<td>acetone, 30min, 40°C</td>
<td>12</td>
<td>61</td>
<td>27</td>
</tr>
<tr>
<td>reference: no PMMA but 2 min O₂ plasma</td>
<td>9</td>
<td>63</td>
<td>28</td>
</tr>
</tbody>
</table>

We observed that by application of the protocol (NMP, 5 min, RT), developed for the S1818 photoresist, we did not properly remove the PMMA coating (40% of C). The best results were obtained by using ultrapure acetone for 30 min with the ultrasonic bath set to 40°C (not stabilized). Under these LO conditions we achieved levels of surface contamination similar to those for a plasma-cleaned niobia surface. Note that the uncertainty on such measurements is estimated to be approximately 5%. Considering this, we cannot completely ensure that no PMMA traces are left on the surface. However, this uncertainty explains the 2% difference seen between Table 4.1 (7% C) and Table 8.1 (9% C).

The survey spectra of the substrates, before and after lift-off, are depicted in Figure 8.2. The spectrum after lift-off was measured on a sample after sonication for 30 min in ultrapure acetone at 40°C.
AFM results

Surface roughness judged by $R_a$ (arithmetic average of the absolute values of all points) was used as a complementary indicator of the PMMA lift-off quality. Table 8.2 lists the average $R_a$ values measured on 1.5x1.5 $\mu$m$^2$ on each type of sample.

Table 8.2  Roughness value, $R_a$ after lift-off under different conditions. All samples were measured after PMMA spin coating, imprinting and lift-off. Analyzed area 1.5x1.5 $\mu$m$^2$.

<table>
<thead>
<tr>
<th>solvent</th>
<th>temperature $[\degree \text{C}]$</th>
<th>time in sonicator $[\text{min}]$</th>
<th>$R_a$ $[\text{nm}]$</th>
</tr>
</thead>
<tbody>
<tr>
<td>NMP</td>
<td>RT</td>
<td>30</td>
<td>0.6±0.1</td>
</tr>
<tr>
<td>NMP</td>
<td>50</td>
<td>15</td>
<td>0.3±0.1</td>
</tr>
</tbody>
</table>
Table 8.2  Roughness value, $R_a$ after lift-off under different conditions. All samples were measured after PMMA spin coating, imprinting and lift-off. Analyzed area 1.5x1.5 μm$^2$.

<table>
<thead>
<tr>
<th>solvent</th>
<th>temperature [°C]</th>
<th>time in sonicator [min]</th>
<th>$R_a$ [nm]</th>
</tr>
</thead>
<tbody>
<tr>
<td>NMP</td>
<td>50</td>
<td>30</td>
<td>0.2±0.1</td>
</tr>
<tr>
<td>acetone</td>
<td>40</td>
<td>30</td>
<td>0.1±0.1</td>
</tr>
<tr>
<td>reference:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>no PMMA+</td>
<td>-</td>
<td>-</td>
<td>0.1±0.1</td>
</tr>
<tr>
<td>2 min O$_2$ plasma</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>

XPS data (Table 8.1) and roughness values (Table 8.2) together indicate that "cleaner" samples (less PMMA residues) have lower $R_a$ values. No significant difference was measured between the reference surface and the surface after lift-off in acetone at 40°C, both have $R_a$ values of approx. 0.1 nm.

8.3.2 Homogeneous PLL-g-PEG adlayer before and after lift-off

XPS analysis

Potential desorption of the functionalized copolymer during lift-off was also evaluated by XPS based on the peak areas of C(1s, PEG) and Nb(3d, Nb$_2$O$_5$) on homogeneous model surfaces (Table 8.3).

Table 8.3  Intensity ratios of C1s (C-O) over Nb3d of PLL-g-PEG coated niobia surfaces before and after lift-off in acetone, 30 min, 40°C.

<table>
<thead>
<tr>
<th>PLL-g-PEG</th>
<th>PLL-g-PEG+lift-off</th>
</tr>
</thead>
<tbody>
<tr>
<td>IC1s (PEG) / INb3d</td>
<td>0.168</td>
</tr>
<tr>
<td>standard deviation</td>
<td>0.010</td>
</tr>
<tr>
<td></td>
<td>0.145</td>
</tr>
<tr>
<td></td>
<td>0.019</td>
</tr>
</tbody>
</table>

The ratio C/Nb is slightly decreased after 30 min in acetone at 40°C. However, this loss is not significant. Also to put this in perspective, the uncertainty in tai-
loring the ligand density by the mixed self-assembly process is estimated to be at least 10%.208

8.3.3 Characterization of the nanopatterns

AFM

Figure 8.3 shows atomic/lateral force microscopy (AFM/LFM) images in contact mode after the window opening by O₂ RIE. The AFM image a) shows that the oxide stripes had a width comparable to the initially imprinted stripes (prior to etching) indicating that the etching was truly anisotropic and did not significantly modify the lateral dimension of the imprinted features. LFM was used (Figure 8.3b) to visualize the chemical contrast between the oxide and the PMMA areas. A relative increase in the friction contrast was revealed after the oxide window opening. The higher friction contrast (185 mV) suggests that the desired chemical contrast of PMMA and Nb₂O₅ was indeed achieved, confirming the window opening. In comparison, the friction contrast at stage III of Figure 8.1 was only 27 mV (image not shown); the fact that this value was not zero is likely a consequence of the stripes topography.
Figure 8.3  a) AFM image in contact mode of the PMMA/oxide topography after O\textsubscript{2} RIE for the window opening (stage IV in Figure 8.1). The imprinted stamp had stripes of 100 nm in width and a period of 400 nm (300 nm spacings). b) LFM image also after etching. The friction contrast has increased from 27 mV (before etching) to 185 mV due to the exposure of oxide stripes by the window opening.

At stage VI of Figure 8.1 we ran AFM scans on the surfaces. The PLL-g-PEG/PEG-biotin stripes were well visible and their width 100±10 nm and period of 408±12 nm correspond to the dimensions of the nanostructured stamp used in the nanoimprinting step (Figure 8.4a). The average step height between oxide and PLL-g-PEG/PEG-biotin (1.5±0.7 nm) is in the range of the thickness of a monolayer as determined by ellipsometry and reported in Section 4.1.2.3.

Figure 8.4  AFM scans (1.5x1.5 μm\textsuperscript{2}) in tapping mode in air of 100 nm patterned stripes. a) PLL-g-PEG/PEG-biotin stripes in an oxide background (after lift-off, stage VI in Figure 8.1). b) After PLL-g-PEG backfill the pattern is still visible due to the longer PEG chains supporting
the biotin molecules (stage VII in Figure 8.1).

To inhibit nonspecific protein adsorption in the background, the Nb$_2$O$_5$ areas were rendered nonfouling by spontaneous adsorption of the non-functionalized PLL-g-PEG from an aqueous solution (“backfilling” step, stage VII in Figure 8.1). The residual step height after backfill (Figure 8.4b) can be attributed to the longer PEG chains (Mw 3.4 kDa) used for the biotinylated poly(ethylene glycol) in comparison to the non-functionalized PEG (Mw 2 kDa). Different PEG chain lengths are generally preferred in surface assembled molecular systems in order to improve the spatial availability and presentation of the bioligand (here biotin) at the interface and reduce the probability of the ligand being embedded in the PEG structure. Also, step height measurements of soft materials should be considered with caution because they are very sensitive to the parameters used and therefore difficult to perform in a reproducible manner.

Patterns of 100 nm wide lines are limited in terms of applications therefore, we explored the possibility to produce arrays of dots in the same size range. Figure 8.5a is an AFM scan of a PMMA film imprinted with a stamp composed of nanopillars of a diameter of 110 nm, distant by 800 nm. After PLL-g-PEG/PEG-biotin adsorption and subsequent lift-off we observed that a number of islands had disappeared. This was likely due to the rupture of several pillars on the stamp during the demolding process and consequently inhibited the window opening (Figure 8.5b). Despite of this mechanical problem, we could show that it is possible to combine nanoimprint lithography (NIL) with MAPL to produce peptide/protein nanopatterns. Reproducibility of the NIL process has still to be optimized in order to produce cm$^2$ areas of individual islands.
Figure 8.5  AFM scans in tapping mode, in air. Image a) shows a PMMA layer imprinted with 110 nm diameter pillars and dry etched with O₂. b) Same sample after MAPL but prior to backfill: PLL-g-PEG/PEG-biotin spots in oxide background. A broken tip was most likely the cause of the "double-features".

An array of 100 nm diameter dots is an adequately-sized platform for applications such as a single vesicle arraying. To immobilize smaller moieties individually, e.g., peptides or proteins, one would have to reduce the spot size down to 10-20 nm. Nanopatterns of such dimensions have been successfully produced elsewhere with nanoimprint lithography (but without chemical functionalization). These patterns would accommodate, in principle, only one protein and allow the investigation of single molecular recognition events.

8.3.3.1 SNOM RESULTS

Scanning near-field optical microscopy (SNOM) was used to image the fluorescent labeled alexa-546 streptavidin adsorbed onto the 100 nm PLL-g-PEG/PEG-biotin stripes (Figure 8.6). The regular line pattern of 400 nm periodicity can be clearly recognized, verifying that streptavidin adsorbed selectively on the PLL-g-PEG/PEG-biotin pattern. The fluorescent-labeled lines appear broader
than 100 nm because the optical resolution was reached. Also, for these experiments pyrex samples (coated with niobia) with a thickness of 500 μm were used. Thinner transparent slides are believed to further increase the resolution of the image.

Figure 8.6 Scanning near field optical microscope (SNOM) analysis of 100 nm biotinylated-PLL-g-PEG stripes decorated with alexa-546 SA in a PLL-g-PEG background. The blurred fluorescent signal from the nanolines is caused by the resolution limit however, the 400 nm periodicity of the grating was respected.

8.3.3.2 CLSM results

For further proof of concept we produced patterns in the micrometer range in the same way as for the nanopatterns. The only differences were that the stamp had microstructures (see design in Figure 8.7a) and that the PMMA layer was 330 nm instead of 125 nm. At the micrometer level, the imprinting technique is more difficult than photolithography in terms of reproducibility. The purpose of this experiment was to be able to analyze the outcome of the imprinting and MAPL with a standard technique such as the confocal microscope (CLSM).

Figure 8.7b is an image of microlines (20 and 50 μm) of labeled streptavidin. The lines displayed an excellent contrast between the biotinylated areas and the
PLL-g-PEG background. No fluorescent signal could be detected on the PEG background.

Figure 8.7  a) Design of the micrometer-featured stamp (PSI, Switzerland). b) Confocal laser scanning microscope (CLSM) image of 20 and 50 μm stripes of biotinylated-PLL-g-PEG in a PLL-g-PEG background. The fluorescent-labelled streptavidin binds specifically to the biotin areas.

8.3.4 Proof of concept for cell-surface interaction studies on nano-MAPL patterns

Single protein/peptide microarray developments are viewed as a great opportunity to unravel a number of fundamental cellular mechanisms involving the extracellular environment. The formation of focal complexes, and later focal adhesions, remain partially mysterious. Nanoarrays with spots composed of thousands, down to a single ligand, with the ability to control the spacings between these features, will accelerate the rate of discovery in the field of cell-surface interactions.

We performed one very basic preliminary tests on MAPL surfaces (micro and nano) that were produced with a combination of NIL and MAPL. Human foreskin fibroblasts (HFF) were plated on a "star-design" micropattern (design:
Figure 8.7a and cells: Figure 8.8a) as well as on 100 nm lines (Figure 8.8b and c) for 48 hours in 10% FBS media.

In all cases we observed that the cells did spread normally and formed focal adhesions as revealed by vinculin spots. The cells spread in a preferential direction as their elongated shape shows in b) and c). Interestingly, the majority of the focal adhesions were elongated along the same direction suggesting that the cells did recognize the orientation of the underlying RGD pattern. These rough observations demonstrate that nanoMAPL patterns can be used in future for more advanced cell assays experiments.
CHAPTER 9

Conclusion and Outlook

This thesis describes the development of a novel chemical patterning technique, named Molecular Assembly Patterning by Lift-off (MAPL), for producing regular (bio)chemical patterns ranging from ~100 nm up to hundreds of micrometers. MAPL combines the advantages of top-down lithography with those of self-organization of functional molecules, and is comparatively simple and cost-effective, not requiring heavy equipment and expensive fabrication steps. MAPL chips with well defined 2D (bio)chemical patterns are able to elicit specific interactions with the biological media. An attractive feature of the MAPL process is its ability to independently and quantitatively control the type/density of bioligands and pattern geometry.

9.1 THE MAPL PROCESS

In the MAPL process, the patterns are transferred from a photolithography mask into a photoresist by UV illumination and development (Figure 3.2). At this stage, the wafer is composed of resist-protected and -unprotected areas. Subsequently, PLL-g-PEG/PEG-X with a fraction of PEG side-chains functionalized with a bioligand X (e.g., biotin or an RGD-containing linear peptide sequence) is
immobilized on the bare (niobia) areas by spontaneous assembly from aqueous solution. The photoresist is then removed with an organic solvent that does not adversely affect the biofunctional PLL-g-PEG/PEG-X adlayer. The resulting surface has patches of PLL-g-PEG/PEG-X in a niobia background. In a final backfill dip-and-rinse step, non-functionalized PLL-g-PEG is adsorbed on the oxide background rendering it resistant to non-specific protein adsorption. Niobium-oxide-coated silicon wafers (or pyrex slides) proved to provide the most consistent pattern quality thanks to negligible polymer loss during lift-off in the organic solvent.

Each step of the MAPL process was characterized by applying complementary surface sensitive analysis techniques (XPS, OWLS, ToF-SIMS, AFM, ELM, CLSM and SNOM). These investigations led to the establishment of optimized MAPL protocols. MAPL is to our knowledge, the first published chemical patterning technology allowing a precise and independent control over island geometry and ligand type and density, while largely inhibiting non-specific adsorption. The surface density of the bioactive molecules is controlled by mixing solutions of functionalized and non-functionalized PLL-g-PEG onto niobium oxide surfaces, requiring the synthesis of only two polymers rather than one for each surface concentration. The PLL-g-PEG and its functionalized derivatives (biotin and RGD) is a well suited polymer since it is highly protein resistant (< 2 ng/cm²) and allows an easy control of the architecture (grafting ratio) as well as the amount of PEG side chains that carry a functional group. Also, this system allows the coupling of virtually any type of bioactive ligand. The polymer was shown to remain stably adsorbed during the lift-off process, provided that the immobilization was performed on a niobium oxide substrate. The simplicity of the MAPL process makes it a cost-effective process allowing the production of micropatterns within a few hours, in constant quality and across relatively large
areas such as 100 mm diameter wafers. Polymer adsorption requires less than an hour and is performed by simple dip-and-rinse steps in aqueous solution. We also believe that the photolithography process could potentially be carried out under "standard" lab conditions, provided there is access to a spin-coater, a heating plate and a mask-aligner outside of a clean room. In terms of simplicity, the MAPL process approaches micro contact printing but the limitations inherent to the two techniques are different; The MAPL samples are "one-use" chips, while the soft stamps can be used repeatedly. For best PLL-g-PEG stability, the pyrex or silicon wafers have to be sputter-coated with a thin layer of transparent niobium oxide. Note, that the process also works on native silicon oxide, TiO$_2$ and ITO but with the restriction that the surface density of the functional groups is not controlled. Spin-coating of photoresist requires a smooth substrate thus, excluding rough and/or complex 3D surfaces. Finally, all spots on a given MAPL chip are composed of the same type and density of bioligand.

9.2 **Cell Patterns**

In chapter 5, we demonstrated that RGD patterns, produced by MAPL, sustain cell attachment and constrain their spreading according to the underlying peptide pattern. Fibroblasts displayed clear focal adhesions and organized actin network when allowed to spread sufficiently. Also, the specificity of the interactions between the RGD motive and the cells was demonstrated. The ability to constrain four different cells lines, suggested that the MAPL platform can potentially be used with any anchorage-dependent cell types.

Time stability of cell patterns, produced by three "in house" techniques (micro-contact printing, SMAP and MAPL), was tested with 3T3 fibroblasts. We found that micro-contact printing on tissue culture polystyrene and MAPL patterns on
niobium oxide were able to constrain cells for at least two weeks. The MAPL patterns with a titanium oxide background displayed cell outgrowth after 24 hours already, whereas the SMAP samples with a silica substrate were stable for seven days. These variations are likely due to differences of negative surface charge densities at the polymer-oxide interface. The loss of pattern fidelity was independent of cellular activity but was attributed to the replacement of PLL-g-PEG by serum proteins.

Cell adhesion strength of murine fibroblasts was compared for fibronectin and RGD modified surfaces with a spinning disk device. Mean adhesion strength on Fn (adsorbed on plasma-cleaned glass) was found to be twice the adhesion strength of the cells on the RGD surfaces. These differences did not correlate with the quantities of available RGD sites (either from Fn or from the synthetic peptide). We hypothesized that the higher adhesion strength was a consequence of the accessibility to multiple types of binding sites on Fn (e.g., synergy or cryptic sites). On the synthetic peptide surfaces, no significant difference in mean cell adhesion strength was measured between 19'000 and 4’800 RGD/μm². It was likely due to the fact that, already the lowest surface concentration presented an excess of RGD ligands compared to an RGD surface inducing maximal adhesion strength.

Here, I would like to take the opportunity to bring this work into another perspective. This thesis focused on cell culture limited to 2D patterned surfaces i.e., flat surfaces without any topography. 2D surfaces have been used for decades to grow cells and study them in vitro. However, in the in vivo case, the tissues are generally formed on a 3D ECM environment and not on flat surfaces. It is known that the 3D environment specifically influences the activity of cancer cells and is crucial for polarization and differentiation of epithelial cells. But what about other cell types commonly forming focal adhesions on 2D surfaces? Aren’t they
merely artifacts of tissue culture? And aren’t we missing biological subtleties? Recently, these questions got increased attention and started to be addressed more systematically. Surfaces that locally provide controlled 3D microenvironment for single cells will be essential for gathering new insights into 3D-related cell mechanisms. The data obtained from 2D surfaces revealed a tremendous number of valuable informations on fundamental cell behavior. At this stand point it is speculated that focal and fibrillar adhesions studied on 2D surfaces represent exaggerated precursors of in vivo 3D-matrix adhesions.

9.3 Protein microarrays

Heterogeneous protein microarrays will, in future, perform as high-throughput tools that will greatly accelerate the understanding of complex biological systems and hence, improved diagnosis and/or prognosis of diseases. The surface properties of such arrays require state-of-the-art surface chemistry for, on one hand, inhibit non-specific interactions and on the other hand, control the bio-ligand immobilization in terms of surface density, orientation and conformation. We performed preliminary experiments to demonstrate the potential of using the MAPL technology for protein microarray applications.

9.3.1 Spotting SA on biotin MAPL chips

In a first set of experiments we defined the best spotting parameters (buffers, concentration, volume) for printing SA on 100x100 μm² biotin patches. The goal was to spot SA tagged with different biotinylated DNA molecules and thus producing a platform for sorting cDNA-tagged lipid vesicles. The alignment of the spots with respect to the biotin patterns was found to be technically difficult. The protocol was shown not to be fully optimized for 25x75 mm² slides; fluores-
cence intensity was measured on the background due to the presence of biotinylated polymer. Upon incubation of a large SA drop, the average intensity variations within a given spot was of 7%. Also, the patterns were found to be highly homogeneous since less than 4% spot-to-spot variation was measured.

9.3.2 PLL-g-PEG/PEG-NTA for protein microarrays

PLL-g-PEG/PEG-biotin and streptavidin were very valuable means to characterize and optimize the MAPL patterns. However, this system suffers two major limitations if one wants to immobilize biotinylated proteins in a subsequent step.286 The highly hydrated and dynamic biotinylated-PEG arms90 are likely to occupy, in a time dependent process, the biotin-binding pockets of streptavidin and thus, inhibit the binding of biotinylated proteins.208 One way to circumvent this limitation is to pre-assemble the streptavidin and the biotinylated molecule in solution before immobilization.207 However, this approach is undesirable for new generation of protein or antibody microarrays. We have performed promising preliminary studies on MAPL patterns with PLL-g-PEG/PEG-NTA chemistry (NTA: nitrilotriacetic acid). The NTA chemistry is highly attractive for such applications since it allows the reversible coupling309 of hexahistidine tagged proteins in the presence of nickel ions.245 Moreover, it is known to provide control over the orientation of the immobilized proteins.119,254 These advantages combined to micropatterns with controlled NTA densities, makes the MAPL an ideal candidate for quantitative protein, peptide, antibody or sugar microarrays.310

9.4 Nanopatterning with MAPL

We have demonstrated that the surface patterning, performed at the microme-
Conclusion and Outlook

The combination of a “top-down” approach via nanoimprinting lithography and a “bottom-up” approach through MAPL was presented as an economical and parallel alternative to other nanopatterning techniques such as dip-pen nanolithography or colloidal lithography. We presented the reproducible immobilization of streptavidin molecules on 100 nm-wide lines of biotinylated polymer separated by PLL-g-PEG. The reproducible production of isolated small feature (e.g., dots) requires further optimization of the stamp mechanical properties as well as the imprinting parameters.

9.5 Outlook

MAPL offers the possibility to study the independent influence of cell spreading, ligand type (single or a mixture of bioligands) and surface density. A systematic study on how these individually controlled surface cues steer the downstream intra-cellular pathways would contribute to a better understanding of fundamental cellular mechanisms. We reported that the culture of arrays of single cells was limited to small patterns (20 μm diameter). To gain additional freedom in terms of spreading of single cells we propose the following setup: the idea would be to produce MAPL patterns with islands of the desired size (spreading area) and then superimpose a flexible stencil composed of holes of the diameter of a cell in suspension (10-20 μm). The membrane (e.g., PDMS) could easily be rendered non-adhesive to the cells with a PLL-g-PEG coating. The challenge would be to align the holes with the underlying cell-adhesive patterns. After 1-3 hours of cell incubation (initial attachment to the underlying adhesive pattern), the membrane could be removed and thus allow the single cells to spread over the defined chemical patterns.
Cell microarrays are also being developed for a number of applications beyond cell-surface interaction studies. For example, Singhvi et al. proposed synchronizing the cells with single cell patterns and thus not requiring pharmacological intervention.\textsuperscript{77} They could also be useful tools for increased rate of production of certain proteins or to stimulate relevant metabolic or secretory pathways.\textsuperscript{311,312} Transfection methods start to benefit from the advantages of cell microarrays: By patterning plasmid vectors, together with cell-adhesive proteins, one can mediate, in a single step, surface transfection or so-called "surffection".\textsuperscript{313,314} Similarly, the need to access high-throughput drug testing on living cells \textit{in vitro} at an early stage of drug development expands the applications of cell microarrays. The low cost and high testing speeds have made cell-based assays a key component of drug discovery programs.\textsuperscript{15,315} For such applications, cell pattern stability may be required for several weeks. Loss of pattern definition with time is directly related to the limited stability of the used polyelectrolyte-based molecular assembly systems, which relies on multiple electrostatic interactions. Desorption at low/high pH values and at high ionic strength as well as slow exchange processes at physiological conditions maybe a limiting factor in certain applications. On the other hand, it is likely that MAPL could be combined with other, more permanent immobilization schemes such as those based on the formation of covalent bonds or strong metal-ion complexation, e.g., with organophosphates/-phosphonates or dihydroxy phenylalanine (DOPA).

The MAPL process was proven to be versatile since it is currently being used within the LSST to generate colloidal patterns (Christoph Huwiler, Brigitte Städler, LSST, ETHZ),\textsuperscript{316} vesicles patterns (chapter 6)\textsuperscript{207} and chemical nanopatterns (Thomas Blättler, LSST, ETHZ). Brigitte Niederberger (diploma thesis) and Marc Dusseiller (both LSST, ETHZ) combined a microfluidic chamber with a MAPL substrate in order to create heterogeneous patterns of vesicles.\textsuperscript{317}
To achieve defect-free patterns on 25x75 mm² microarray slides for spotting, minor optimization are still needed, namely preventing the re-adsorption of biotinylated polymer in the background. As a potential solution we propose an alternative to the NMP rinsing: the idea would be to plunge the sample in a bath of NMP mixed with water in order to slow down the dissolution kinetic and thus, only remove the first nanometers of PR together with its bound functionalized PLL-g-PEG. In a subsequent step, the sample would be dipped into a pure NMP solution to completely remove the PR. Finally, the bare oxide would be passivated, as usual, with a pure coating PLL-g-PEG.

To improve the reproducibility of 100 nm spots there is a need for further optimization of the stamp mechanical properties. We are confident that by decreasing the island size below 100 nm it will be possible to produce arrays of single peptide/proteins. A study based on NIL reported sub-10 nm feature sizes. The smallest island achievable with this system is probably limited by the size of the PLL-g-PEG molecule used in this work (footprint of approximately 70 nm). In an effort to pursue this pattern "down-sizing" we have recently synthesized a polymer with lower PLL molecular weight in order to accommodate the molecule on smaller nanopatterns (Brigitte Städler and Stéphanie Pasche, both LSST, ETHZ).

9.6 Final remarks

Progresses on the developments of novel nano and micro biochemical patterning techniques are growing at rapid pace. But these impressive technological achievements are not a goal per se. Their main purpose is to provide new tools for a better understanding of biological phenomenon as well as supporting inno-
vative applications in biotechnology. A more widespread acceptance and accessibility is necessary for implementing these young technologies in solving specific and well-defined biology-related questions. The parallel emergence of new analytical methods allowing to study single molecules or individual living cells (e.g., molecular beacons) combined with state-of-the-art biochemical patterns, will in a short future, tremendously contribute to deciphering mechanisms of diseases/healing and, on the long term, improve human health and well-being. It seems clear that the collaborative effort of scientists from highly diverse fields is a key requirement for any success in biosciences nowadays. Also the results and their potential implications on the society have further widen the spectrum of specialists to include for example ethicists. This necessary approach permits to elevate the debate to a non-purely scientific level and thus bring the crucial aspects into other perspectives.
10.1 MATERIALS AND PROTOCOLS

10.1.1 Substrate materials

Silicon wafers <100> (Wafernet) of a diameter of 100 mm with two orthogonal "flats" and a thickness of 525±25 μm. Pyrex 7740 (glass) wafers (SensorPrep Services, USA) of the same dimensions were used as transparent substrates (Ra < 15 Å). Low auto-fluorescent borosilicate float glass slides (25x75x1 mm³) from Schott (Schott-Nexterion, Glass D) were received as a kind gift from Jens Sobek (FGCZ). All types of substrates were either used as received (opened in the cleaned room to avoid any additional cleaning process) or sputter coated (no cleaning) with a Leybold dc-magnetron Z600 sputtering plant located at Paul Scherrer Institute (Villigen, Switzerland). Oxides coatings between 10 and 20 nm thickness of Nb₂O₅ were deposited. They were then further processed in the clean room (Section 10.1.3) without additional cleaning.
10.1.2 PLL-g-PEG and its functionalized derivatives

PLL-g-PEG synthesis of batch SP09 was performed by Stéphanie Pasche (LSST, ETHZ) by reacting poly(L-lysine) hydrobromide (PLL-HBr) (Mw 27'400 Da) and succinimide propionate methoxy-PEG (mPEG-SPA) (Mw 2 kDa) at a molar ratio corresponding to a grafting ratio (g) of 3.3. All synthesis details can be found in. PLL-g-PEG/PEG-biotin (50%) was synthesized by Firat Durmaz (LSST, ETHZ) by reacting poly(L-lysine) hydrobromide (PLL-HBr) and N-hydroxysuccinimide PEG (biotin-PEG-NHS) functionalized with biotin. The mPEG-NHS were added upon reaction completion of the biotin-PEG-NHS. Note that the new synthesis use PEG-SPA instead of PEG-NHS for enhanced stability against hydrolysis. PLL-g-PEG/PEG-RGD (6.5%) was synthesized by Martin Schuler (LSST, ETHZ) in accordance to the protocol published by Vandevondele et al.160 In brief, PLL, NHS-PEG (Mw 2 kDa) and NHS-PEG-VS (Mw 3.4 kDa) (VS=vinylsulfone) were predissolved individually in HEPES-buffered water (pH 8.4) and mixed in a ratio of one PEG chain per 3.5 lysine monomers. The amount of NHS-PEG-VS was dependent of the desired ratio of functionalized PEG chains. The peptide, N-Acetyl-GCRGYGRGDSPG-amide, was added in a 2-fold molar excess of the VS groups. The peptide was synthesized using standard FMOC-chemistry.

The lyophilized polymer, in form of a white powder, was then stored at -20°C until use. The polymer was solubilized in 4-(2-hydroxyethyl)piperazine-1-ethane-sulfonic acid (HEPES) buffer (Fluka, Buchs, Switzerland) adjusted to pH 7.4 with 6M NaOH (Fluka, Buchs, Switzerland) + 150 mM NaCl (Hepes 2). The concentration of the polymer solution was 0.1 mg/ml. The reason to use Hepes 2 is its physiological salt concentration. Moreover, if the polymer is adsorbed in Hepes 1 (10 mM, no NaCl) we observe a desorption (~10%) when the media is
changed to a 150 mM solution. Since the growth media for cell culture is at 150 mM we decided to use Hepes 2 for all adsorptions. The polymer solution was filtered before use with a 0.22 μm filter. The solutions were stored for a maximum of 3 weeks at 4°C.

Table 10.1 and Table 10.2 provide the molecular weights of the molecules of interest.

**Table 10.1** Monomers molecular weight.  

<table>
<thead>
<tr>
<th>Molecule</th>
<th>Molecular weight [Da]</th>
<th>denomination</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lys-HBr</td>
<td>209</td>
<td>M_{lys-HBr}</td>
</tr>
<tr>
<td>Lys</td>
<td>128</td>
<td>M_{lys}</td>
</tr>
<tr>
<td>EG</td>
<td>44</td>
<td>M_{EG}</td>
</tr>
<tr>
<td>biotin</td>
<td>244</td>
<td>M_{bio}</td>
</tr>
<tr>
<td>peptide</td>
<td>1080</td>
<td>M_{RGD}</td>
</tr>
</tbody>
</table>

**Table 10.2** Polymers molecular weights. Note that the "long" PEG chains were used exclusively to couple functional groups such as biotin or peptides.

<table>
<thead>
<tr>
<th>Polymer</th>
<th>Molecular weight [Da]</th>
<th>denomination</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLL-HBr</td>
<td>~27’000</td>
<td>M_{PLL-HBr}</td>
</tr>
<tr>
<td>&quot;Short&quot; PEG</td>
<td>1885</td>
<td>M_{PEG}</td>
</tr>
<tr>
<td>&quot;Long&quot; PEG</td>
<td>3041</td>
<td>M_{PEG}</td>
</tr>
<tr>
<td>&quot;Long&quot; PEG-biotin</td>
<td>3285</td>
<td>M_{PEG-bio}</td>
</tr>
<tr>
<td>&quot;Long&quot; PEG-peptide</td>
<td>4120</td>
<td>M_{PEG-RGD}</td>
</tr>
</tbody>
</table>

Based on adsorbed polymer mass (OWLS), the grafting ratio g (NMR), and fraction of functionalized PEG chains, the calculation of the PEG-biotin (or
PEG-RGD) surface density becomes straightforward.\textsuperscript{90}

The number of PEG chains per nm\textsuperscript{2} for a pure PLL-g-PEG coating is given by Equation 10.1.\textsuperscript{90,\textsuperscript{91}}

\[
n_{\text{PEG}} = \frac{m_{\text{pol}}}{M_{\text{lys}} \cdot g + M_{\text{PEG}}} \quad \text{Equation 10.1}
\]

The number of biotin (or RGD) per nm\textsuperscript{2} for a pure PLL-g-PEG/PEG-biotin coating is given by Equation 10.2.

\[
n_{\text{PEG-bio}} = n_{\text{lys}} \cdot \frac{x}{g} \quad \text{Equation 10.2}
\]

where the number of lysine per nm\textsuperscript{2} (\(n_{\text{lys}}\)) is given by Equation 10.3.

\[
n_{\text{lys}} = \frac{m_{\text{pol}}}{M_{\text{lys}} + \left(\frac{1-x}{g} \cdot M_{\text{PEG}}\right) + \left(\frac{x}{g} \cdot M_{\text{PEG-bio}}\right)} \quad \text{Equation 10.3}
\]

Note that the \(m_{\text{pol}}\) is in g/cm\textsuperscript{2} and that the M are in Da (=g/mol) resulting in a surface density expressed in mol/cm\textsuperscript{2}. To convert it into molecules (PEG, biotin or RGD) per cm\textsuperscript{2} (or nm\textsuperscript{2}) simply multiply by Avogadro’s number: \(6.02 \times 10^23\).

For a mixed assembly of biotin (or RGD) functionalized and non-functionalized coating the expression describing the biotin surface density, in mol/cm\textsuperscript{2}, is given by Equation 10.4. This equation assumes that both polymers have the same chain length, adsorption kinetics and stability i.e., that in an equimolar mixture the number of lysine monomers on the surface are identical for both polymers.
Complementary information on materials, protocols and instrumentation

\[ n_{\text{PEG-bio}} = n_{\text{lys(tot)}} \cdot y \cdot \frac{x}{g_{\text{funt}\cdot\text{pol}}} \]  
*Equation 10.4*

where \( y \) is the molar fraction of the functionalized polymer (\( y = \frac{n_{\text{lys(funct,poly)}}}{n_{\text{lys(tot)}}} \)); \( x \) the fraction of PEG-biotin (or RGD) chains on the functionalized polymer \( g_{\text{funt,pol}} \) the grafting ratio of the functionalized polymer. The total number of lysine monomers on the surface (\( n_{\text{lys(tot)}} \)) is given by Equation 10.5.

\[ n_{\text{lys(tot)}} = \frac{m_{\text{pol(mixed)}}}{M_{\text{lyt}} + y \cdot \left( \frac{M_{\text{PEG}}}{g_{\text{pol}}} \cdot \frac{1}{y} \right) - \left[ \frac{(1-x)}{g_{\text{funt}\cdot\text{pol}}} \cdot M_{\text{PEG}} \right] + \left[ \frac{x}{g_{\text{funt}\cdot\text{pol}}} \cdot M_{\text{PEG-bio}} \right]} \]  
*Equation 10.5*

### 10.1.3 Photolithography

The following steps were carried out in the clean room of prof. Baltes (PEL, ETHZ) with a Electronic Vision Al6-2 mask aligner (mercury lamp 355nm, 405nm, 436nm) in contact mode. The glass/chromium masks were designed with AutoCAD 2000 and manufactured by Delta Mask in the Netherlands (www.deltamask.nl). For detailed informations about microlithography we refer the reader to the excellent manual published on the web by Cornell University.\textsuperscript{150}

Blow dust particles away for wafer surface; evaporate surface moisture on hot plate 115°C, 2 min; wait 1min for cooling (should not be done on silicon wafers because the final photoresist adhesion will be too weak and delamination in Hepes 2 will occur); spin coat positive Shipley S1818 photoresist, \( \sim 2 \) ml at 4000
rpm (use a small chuck for microarrays slide or smaller substrates, note that for thick substrates first check the vacuum and then add PR. For 1 mm thick slides press on the slide to reach vacuum); soft-bake wafer 115°C, 2 min to evaporate the solvents; after 1 min cooling, insert wafer in mask aligner; illuminate for 10 to 15 s; develop for 45 to 60 s with gentle shake (mircroposit 351 1:5 v/v H₂O); rinse under ultrapure water ~5 min (if shorter rinsing time is applied, delamination is likely to occur) or put in a large volume of ultrapure water; spin-dry, do not let dry the drops; check microstructure quality under microscope (this is very important since small variations were observed from time to time) and/or with the alpha-stepper. Note that this protocol works reproducibly for structures down to 3-5 μm and the resulting PR thickness is ~1.5 μm. To achieve 1 μm lateral resolution the photoresist has to be diluted 1:1 with microposit EC solvent and illumination and development times needed to be reduced to respectively, 7 s and 20-30 s.

For spotting slides (25x75 mm²) it is important that the patterns are aligned (parallel) to the slide edges. Since the mask aligner used was not configured for such alignment purposes we used the following trick: we inserted the mask into the device, placed a photoresist-coated 100 mm diam. silicon wafer in contact and illuminated and developed it. Then we repositioned the PR-patterned wafer onto the wafer chuck (precisely in the same position as in the precedent step) and placed the PR-coated microarray slide on top of the wafer and align it according to the underlying patterns (visible by eye). We designed a mask specifically for protein microarrays (mask name MC7) composed of squares of 100x100 μm² with a center to center distance of 400 μm.
Complementary information on materials, protocols and instrumentation

**Figure 10.1** Major components of the Shipley S1818 photoresist.

Note that the same photoresist is available with increased adhesion (S1818 SP-16). However, this has to be taken into account during the lift-off step.

The wafers were finally cut (out of the clean room) with a wafer dicing machine (Kulike and Soffa) into 1x1 cm² samples with continuous water flow (set to max. level). It is important that the wafer is well taped on the adhesive foil (avoid air bubbles) otherwise, the samples will be ejected in the dicing process.

Note that the cutting blades for silicon and pyrex are different as well as the cutting parameters and program "100" should be used for silicon and "100Glass" for Pyrex.
10.1.4 MAPL protocol

Adsorption of the functionalized PLL-g-PEG

1. mark the sample (on glass substrates) with scratches to identify the active side. 2. put 1 sample into a piranha-cleaned glass beaker (vol. 3 ml) (sample stand upright). 3. 5 min with ultrapure water in ultrasound bath (put the beaker into a large glass container partially filled with water). 4. rinse samples under Millipore water flow, N2 blow-dry. 5. 10 s O₂ plasma (2.10⁻² bar) to remove surface contamination (knob on "high" on PDC32G Harrick plasma cleaner). 6. place samples into TCPS well plates or on a sheet of parafilm. 7. adsorb PLL-PEG (+biotin, RGD, etc) for 40 min in Hepes 2 (0.1 mg/ml). I usually just deposit a drop covering the whole sample surface, it also as the advantage not to coat the backside with functionalized polymer and not to use extensive amount of polymers. This is especially true when 25x75 mm² microarrays slides are to be coated. 8. rinse the samples under millipore H₂O. 9. blow-dry with nitrogen (important, otherwise you will transfer functionalized polymer into the next beaker!). Note that for PMMA nanopatterns the oxygen plasma step is not necessary if adsorption if performed immediately after reactive ion etching.

Lift-off

The Lift-off process has to be carried out under an aspiration hood!

1. fill glass beaker with 3.5 ml NMP (N-methyl pyrrolidone for peptide synthesis, Fluka, ref 69116). 2. hold sample on edge (tweezer) tilt the sample with a negative angle and flush with NMP (in squeeze bottle) (3 s by counting) to partially remove PR and the functionalized PLL-g-PEG on it. If you do not do that the PLL-g-PEG/PEG-X that was on the photoresist will be in the NMP beaker and thus adsorb on the background, where the resist was and hence decrease the pattern quality! (see Section 5.4) 3. put sample immediately into NMP beaker
and in ultrasound, do it fast because if surface dries it will not be possible to remove photoresist! (the beaker should be in a larger container with a little water to transmit the ultrasonic waves). For microarray slides use several NMP-filled 140 ml glass containers. 4. after 30s remove 1ml NMP (pink color) and refill with fresh NMP and put back in ultrasonic bath for 1 more min (do this with the dedicated micro-pipette!) 5. transfer sample into a beaker full with fresh NMP for 5 min (in ultrasound) 6. transfer the sample into a beaker containing 1ml H₂O and 1ml NMP for 1 min in ultrasound. 7. from point 3-5, 7 min have past, transfer the samples into an ultrapure water bath stirred by a magnet for 5 min. 8. rinse under Millipore flow, dry with nitrogen stream. Immediately proceed to the backfilling step, otherwise the background will be contaminated with adventitious particles. 9. Dispose of the NMP into the organic solvents waste container!

Note that for the nanoimprinted PMMA patterns, ultrapure acetone was used for 30 min in the sonicator at 40°C.

![Figure 10.2 N-methyl pyrrolidone (NMP); C₅H₉NO.](image)

Note that we also tried a commercial photoresist stripper developed specifically to lift-off S1818 photoresist namely, Shipley SVC-175. We did not quantify the lift-off efficiency but instead carried out the whole MAPL process. The patterns were qualitatively similar to the NMP based lift-off protocol.
PLL-g-PEG backfill

1. adsorb non-functionalized PLL-g-PEG for 40 min. 2. Millipore rinse, N₂ blow-dry, store in box in air or argon atmosphere at room temperature. We have observed that stored in air the PLL-g-PEG retains its protein resistance capability for at least 6 months (see protein microarrays results).

Streptavidin adsorption

Alexa-labeled streptavidin (SA*) was used (Molecular Probes) in many experiments of this work. Except in spotting experiments, the solutions used were 20 μg/ml (0.3 mM) in Hepes 2 and incubated in dark for 30-60 min. The surfaces were then rinsed with Hepes 2 buffer and with Millipore water, before being nitrogen blow-dried and stored in the dark. Note that the Alexa dyes are extremely bright and stable and can thus be handled for hours at normal day light if not used for quantitative measurements.

Cell patterning

Since the whole MAPL surface modification takes place in the lab one should be aware of possible bacterial contamination. We very rarely observed bacteria colonization on the cell patterns. However, such events are mainly dependent on the handling skills of the scientist as well as the care put into the sample production (tweezers cleanliness, 0.22 μm filtered solutions, adsorption in laminar hood, etc). Note that for more security it is possible to rapidly rinse the final MAPL surfaces with 70% ethanol, ultrapure water and nitrogen blow-drying prior to cell seeding. Note that this step was generally not performed in this study.

10.1.5 Protein microarray slides

Microarray slides of 25x75 mm² and a thickness of 1 mm (manufactured by Schott and received by Jens Sobek, FGCZ, ETHZ-UNIZH). We sputter coated
the slides as received with 12 nm of niobium oxide. To keep the surface clean we only opened the slide container under a laminar flow hood. Markings were made on each slide with a glass-writer in order to identify the coated side (do not use water-resistant pen). The x-y coordinates of the first pattern were measured under a light microscope equipped with a scale in the binoculars. Note that the quality of the photoresist pattern and the alignment of the islands can be checked at this time. The MAPL process (clean-room and chemical functionalization) was then performed as described in Section 10.1.3 and Section 10.1.4.

Spotting solutions and conditions, washing and evaluation are discussed in chapter 7.

10.1.6 NanoMAPL

Nano-imprinting was performed by Konrad Vogelsang on a "home-build" embossing device at Paul Scherrer Institute (PSI, Switzerland). For reactive ion etching we used a STS dry etcher (at PEL, ETHZ, Prof. H. Baltes) with the following parameters: power: 20 W; chamber pressure: 20 mTorr; O₂ flow: 20 Sccm; bias: 189 v; time: 2 min 45 s. After etching the sample topography was checked with an alpha-stepper. Further experimental details are described in Chapter 8. Note that based on the results from Table 8.1, we believe that NMP could potentially also be satisfyingly applied as lift-off solvent, provided some optimization of the processing parameters. Other lift-off solvents have been used and published elsewhere.³¹⁸,³¹⁹

10.1.7 Cell culture and immunofluorescent stainings

Four different cell lines were used in this work, namely human foreskin fibroblasts (HFF), 3T3’s fibroblasts (expressing GFP–labeled β₃-integrin, source: B.
Wehrle-Haller, University of Geneva, Switzerland), NIH 3T3’s fibroblasts and MG63 human osteosarcoma cells. We followed culture conditions recommended by the cell suppliers. Note that the following abbreviations will be used in this paragraph: FBS: foetal bovine serum (Hyclone, USA); NCS: new born calf serum; DMEM: Dulbecco’s modification of eagle’s medium (Cellgro, USA); PBS: phosphate buffered solution. All products were heated to 37°C in a water bath prior to use.

**HFFs and 3T3’s fibroblasts:** cells were removed from the culture flasks at 80-90% confluency and after a PBS wash and addition of a trypsin/EDTA solution (0.25% trypsin, 1mMEDTA) for 3-5 minutes in the incubator. Note that 2.5 ml of trypsin solution are sufficient for T75 flasks that generally comprise 4-8 million cells however reduce the necessary time to a minimum since the trypsin unspecifically cleaves amino-acids. The cell suspension was diluted with 10% FBS containing medium to inactivate the trypsin (15 ml for T75 flask) and centrifuged at 1100 RPM for 5 minutes. Subsequently the sedimented cells were resuspended in DMEM and centrifuged again. After resuspension in DMEM the cell concentration was determined with a hematocytometer (50 µL cell suspension + 50 µL trypan blue (toxic) count cells in 8 different areas of the grid) and the desired amount of cell suspension diluted in the medium selected for plating. The plating density used was always between 5’000 and 30’000 cells/cm² and the medium contained 10% FBS and 1% antibiotics/antimycotics (penicilin and streptomicin) solution. No protein preadsorption was done before cell plating unless otherwise mentioned. All cell culture reagents were obtained from Gibco BRL (Life Technologies, Rockville, USA) unless otherwise mentioned. **MG63 osteosarcoma cells:** The MG63 cell line was originally derived from a human osteosarcoma. The cells exhibit characteristics of relatively immature osteoblasts and were provided by the lab of Dr. Boyan. The same culture protocol was used
as described above except that no EDTA was used to detach the cells from the flask. For Murine NIH 3T3’s fibroblasts the FBS was changed to 10% NCS.

**Immunofluorescent stainings for focal adhesions (vinculin); cytoskeleton (F-actin) and nuclei (DNA)**

We strongly recommend to the experimenter to look carefully at the safety data sheets provided by the suppliers. This is especially important for engineers that are less used to work with biohazard materials, some chemicals are extremely carcinogenic (for ex. formaldehyde) and require great care.

For optimal staining quality (also for integrin stainings) we recommend the following protocol (developed by the group of A. Garcia, GeorgiaTech, USA). Other, more simple, protocols are available elsewhere and are providing good results as for example on fibroblasts.

1. Permeabilize cells in ice-cold 0.5% Triton X-100 in CSK (cytoskeleton stabilizing buffer) for 3-5 min. 2. Fix cells in 3.7% formaldehyde (PBS) for 4 min. 3. Incubate in BB (blocking buffer: 5% FBS + 0.1% Tween-20 + 0.05% Sodium azide) for 1 hour at 37°C. Note that we experienced problems with tween-20 and may be a cause of cell detachment. 4. Incubate mouse anti-vinculin primary antibody with a drop of 40 μl sandwiched between the sample (upside-down) and parafilm for 1 hour at 37°C. Dilution of primary antibody (anti-vinculin) from stock solution in BB with a ratio of 1:300 (mouse monoclonal IgG from Upstate, USA). 5. Wash 3x with PBS thoroughly. 6. Incubate drop of 40 μl of secondary antibody (light sensitive!) diluted in BB for 1 hour at 37°C. Secondary antibody is conjugated to alexa 488 fluorescent dye and directed against the IgG species of the primary antibody (goat-anti mouse alexa 488 IgG from Molecular Probes). In addition you may add Hoechst (or DAPI at 3 μM) for nuclei staining and/or rhodamine-phalloidin for F-actin (dilution 1:200). 7. Wash 3x thoroughly. 8.
Rinse with ultrapure water. 9. Mount using mounting media, keep dark overnight on a flat surface and seal with nail polish. 10. Store in the dark and for long-term storage keep at 4°C.

**CSK buffer:** 50 mM NaCl, 150 mM sucrose, 3 mM MgCl₂, 50 mM tris(hydroxymethyl)aminomethane, pH 6.8, 20 μg/ml aprotinin, 1 μg/ml leupeptin and 1 mM phenylmethylsulfonyl fluoride (PMSF). Note that aprotinin, leupeptin (both are protease inhibitors from Sigma) and PMSF should be added to the buffer prior to each experiment since these compounds are only stable for 3 hours after thawing.

For DiI membrane staining add 5 μL/well to growth media of stock solution (1.5 mg/ml) for 30 min at 37°C.

**Live/dead assay**

The live/dead assay kit from Molecular Probes (kit L 3224) was used according to the supplier’s recommendations. This cell viability (necrotic and apoptotic) test provides indications of the membrane integrity and the esterase activity.

**Spinning disk assay**

Murine NIH 3T3’s fibroblasts were cultured in DMEM + 10% NCS for 16 hours. Centrifugation was performed in 2 mM dextrose (low viscosity) and 5% dextran (500 kDa) in PBS. Remaining cells were then fixed in 3.7% formaldehyde and nuclei stained with ethidium homodimer for 30 min at 37°C. Finally, the samples were rinsed with PBS and analyzed under a x-y motorized microscopy stage.
10.2 Instrumentation and Parameters

10.2.1 Ultra-high vacuum techniques

X-ray Photoelectron Spectroscopy (XPS)

XPS spectra were recorded at a 90° detector angle with a SAGE 100 system (Specs, Berlin, Germany) using nonmonochromatized Al Kα radiation at 320 W (13 kV) and an electron-energy analyzer pass energy of 50 eV for low resolution surveys and of 14 eV for high-resolution detailed scans. The analyzed area was 6 mm² with a typical information depth of ~10 nm. To determine the quantitative surface composition from XPS data, Scofield’s sensitivity factors were used and are shown in Table 10.3. The peak fitting was performed with CASA XPS software (Ver. 2.2.24) and the energy given as the electron binding energy.

<table>
<thead>
<tr>
<th>Element</th>
<th>Sensitivity Factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nb 3d</td>
<td>8.21</td>
</tr>
<tr>
<td>C 1s</td>
<td>1</td>
</tr>
<tr>
<td>O 1s</td>
<td>2.93</td>
</tr>
<tr>
<td>Ti 2p</td>
<td>7.81</td>
</tr>
<tr>
<td>Si 4f</td>
<td>0.82</td>
</tr>
</tbody>
</table>

Note that the following points were taken into account for the curve fitting procedure. The spectra were calibrated with the C-C binding at 285.0 eV. The full width at half maximum (fwhm) was constrained between 1.5 and 1.7 for all 1s peaks. The Gauss to Lorenz ratio was set to 60 for all 1s peaks and 30 for 3d. The fitting of the peaks for the different carbon components was aided by the theoretical values for a layer of PLL(20)-3.5-PEG(2) on niobia: (C-C: 12.2%, C-O/C-N: 78.6%, C=O: 3.1%) (personal communication from Laurent Feuz, LSST, ETHZ).
Time-of-Flight Secondary Ion Mass Spectrometry (ToF-SIMS)

Secondary ion mass spectrometry (SIMS) is based on the bombardment of a sample surface with primary ions, generating the emission of secondary species (Figure 10.3). This sputtering process can be described as a collision cascade where energy is transferred to molecules at the outermost surface. These collisions lead to fragmentation and bond breaking, and to the emission of positive, negative and neutral molecular fragments, with an initial kinetic energy ranging from 1 to several hundred eV.\(^{210}\) The SIMS is considered static below an ion dose of typically \(10^{12}\) to \(10^{13}\) ions/cm\(^2\) and dynamic above because of the extensive surface sputtering (used for depth profiling). Static SIMS is suitable for the analysis of the surface of materials, due to its high surface sensitivity with an information depth close to 1 nm and a detection sensitivity of \(10^7\) to \(10^{11}\) atoms/cm\(^2\) (10\(^{-6}\) to 10\(^{-2}\) monolayer).

Figure 10.3  ToF-SIMS principle.
The chemical mapping and the spectra recorded in this thesis were performed on a PHI 7200 time-of-flight secondary-ion mass spectrometer under static conditions and a chamber pressure of $< 2.10^{-8}$ Bar. Spectra were recorded with an 8 kV Cs$^+$ primary ion beam (200 μm beam diameter) and with the same parameters as Stéphanie Pasche (LSST, ETHZ). Imaging was performed with the In$^+$ liquid metal ion gun (LMIG). In both cases the substrates were silicon wafers coated with 10-20 nm niobium oxide. The use of pyrex/glass substrates should be avoided because of significant charging effects and loss of mass resolution.

10.2.2 Optical techniques

Optical Waveguide Lightmode Spectroscopy (OWLS)

With the optical waveguide lightmode spectroscopy (OWLS), the adsorbed mass is calculated from the change of the refractive index in the vicinity of the surface upon adsorption of molecules from solution. This change is monitored by the grating induced incoupling of laser light into the waveguiding substrate, generating an evanescent wave (~100 nm into the media). For each polarization mode of the light (transverse electric, TE, and transverse magnetic, TM) there is a discrete incoupling angle $\alpha$ corresponding to maximum constructive interference. From the changes in incoupling angles for TE and TM upon molecular adsorption, the thickness and effective refractive index of the adlayer were calculated. The sensitivity of the OWLS technique is typically 1-2 ng/cm$^2$.90
Figure 10.4 OWLS setup with a flow-through cuvette mounted on the top of the waveguide. The whole system is rotating left and right in order to scan the incoupling angle α, and the incoupled light is detected with a photodiode on each extremity of the waveguide.

The dn/dc value used for the different PLL-g-PEG polymers was 0.158 cm$^3$/g. The published value of 0.182 cm$^3$/g was used for all protein adsorption quantifications. This value was then used to calculate the adsorbed mass of polymer (or protein) $M$, according to de Feijter’s approximation (Equation 10.6).

\[ M = \frac{n_A - n_C}{d_n/d_c} \cdot d_A \]

Where $n_A$ and $n_C$ are respectively, the refractive indexes of the adsorbed layer (polymers or proteins) and the covering medium. $d_A$ is the thickness of the adlayer and $d_n/d_c$ is the refractive index increment with concentration [cm$^3$/g]. Note that the value of 0.158 cm$^3$/g chosen for all polymers is arguably not very rigorous (experimental value obtain with the refractometer) and we suggest for further studies to use the theoretical values proposed by Stéphanie Pasche (LSST, ETHZ).
Confocal Laser Scanning Microscope (CLSM)

CLSM allows to scan the sample surface with a laser and measure fluorescent signal originating from the chromophores. In addition to the scanning, the confocal setup enables the reduction of the focal plan to a thickness of \(\sim 100\) nm by excluding, via a pinhole, the light coming out of the defined focal volume (Figure 10.5). The Photomultiplier (PMT) enhances the photon signal by a cascade process prior to converting the light into electrical signal and feed into the computer for image reconstruction.

The microscope used in this study was a Zeiss LSM 510. High resolution images and quantitative investigations were carried out with oil immersion objectives. The standard procedure to determine the dynamic working range of the microscope was to locally bleach repeatedly (100% laser power) until the signal would be stabilized to a minimum. The detector gain and amplifier offset were adjusted so that the "zero" signal (bleached area) could be set to \(\sim 3\) (rel. intensity) and the highest signal close to 250 (for an 8 bit data acquisition mode).

Figure 10.5  CLSM principle. Source: http://www.zeiss.com
Microarray slide scanner

The microarray slide scanner works in the same way as the CLSM. A GSI Lumonics scanner was used (located in the Functional Genomics Center, Zürich-Irchel) with slides of 25x75 mm². Such scanners are optimized for these specific use and are thus extremely sensitive over a broad dynamic range. Moreover, it has a 16 bit acquisition system dividing the full intensity range into 65,535 levels. Image analysis was performed with the freely accessible software ImageJ ver. 1.32J (http://rsb.info.nih.gov/ij).

Spectroscopic Ellipsometry (ELM)

Ellipsometry is based on the change upon polymer (or protein) adsorption of the state of polarization of elliptically polarized light reflected at a planar surface. From the changes in the ellipsometric angles the refractive index and the thickness of the film can be deduced. The measurements and evaluations typically are done within minutes, provided that an adequate layer model is available. The disadvantages with the method include the requirement of reflecting surfaces (we used niobia coated silicon wafers), and the rather complex theory in cases when extremely detailed information is required or if systems with unknown optical properties are investigated.

Scanning Nearfield Optical Microscopy (SNOM)

The Scanning Near-Field Optical Microscope (SNOM) is a scanning probe microscope that allows optical imaging with spatial resolution beyond the diffraction limit. A nanoscopic light source, usually a fiber tip with an aperture smaller than 100 nm, is scanned very closed to the sample surface in the region called "optical near-field" (source www.csem.ch). Light passes through a sub-wavelength diameter aperture and illuminates a sample that is placed within its near field, at a distance much less than the wavelength of the light Figure 10.6.
The resolution achieved is far better than that which conventional optical microscopes can attain (http://www.nanonics.co.il).

Figure 10.6 SNOM principle. Source: http://www.csem.ch

The achievable optical resolution is mainly determined by the aperture size of the optical probe and the probe-surface gap. A SNOM instrument must therefore feature: i) feedback system keeping the probe at constant distance from the surface. As a benefit, this allows to record the samples topography simultaneously and ii) a high quality near-field probe.

Rolf Eckert (CSEM, Switzerland) performed the SNOM measurements (Figure 8.6) with a "home-build" instrument. The sample thickness was 500 μm (Pyrex coated with 12 nm niobia). Thinner slides are likely to provide higher resolution.

Atomic Force Microscopy (AFM)

A Nanoscope IIIa-MultiMode AFM (Digital Instruments, USA) was used in air in tapping mode in order to visualize the PLL-g-PEG/PEG-biotin patterns on the bare niobia substrates. The piezo actuator transmits its excitation frequency
(\sim 200 \text{ kHz}) \text{ to the SiO}_2 \text{ tip. The natural oscillating frequency of the tip is constant throughout the measurements, the information is gained by the amplitude changes due to the surface chemistry. These changes are recorded by the laser beam deflection on the photodiode. In lateral force microscopy (LFM), also called friction mode, the lateral torsion of the cantilever is recorded.}
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Didier Falconnet
Date of birth: April 11th, 1976
Nationality: Swiss, Citizen of Vuisternens-en-Ogoz (FR)
Present address: En Bellanger, CH-1271 Givrins (VD)

Education

2001-2005 Swiss Federal Institute of Technology, ETH-Zürich
Laboratory for Surface Science and Technology
(Prof. Dr. M. Textor, Prof. Dr. N. D. Spencer)
Department of Materials
Ph.D. Student

2004 Georgia Institute of Technology, Atlanta, Georgia, USA
(Prof. Dr. B. Boyan, Prof. Dr. G. Bao)
Visiting Scholar for 5 months

1996-2001 Swiss Federal Institute of Technology, EPF-Lausanne
Master in Material Sciences
07/00-03/01 Diploma Thesis at the Katolieke Universiteit Leuven (KUL), Belgium
(Prof. Dr. I. Verpoest, Dr. P.-E. Bourbon, Prof. Dr. J.A.E. Manson)
"Mode I interlaminar fracture toughness of glass knitted textile composites"

1991-1995 Gymnase Cantonal, Nyon
"Maturité Type C"
Publications

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D. Falconnet; A. Koenig; F. Assi; M. Textor.
*Journal cover page; fibroblast cell confined to an RGD square micropattern.

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**D. Falconnet; D. Pasqui; F. Assi; A. Koenig; R. Barbucci; M. Textor.**

**D. Falconnet; D. Pasqui; F. Assi; A. Koenig; M. Textor.**
AVS annual meeting, Nov. 2-7, 2003, Baltimore, USA.

**D. Falconnet; D. Pasqui; F. Assi; A. Koenig; M. Textor.**
Kolloquim D-MATL, October 22, 2003, ETH Zürich, Switzerland.

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