New Micropatterning Techniques for the Spatial Addressable Immobilization of Proteins

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ABSTRACT

Bio-microdevices are miniaturised devices based on biologically derived components (e.g., DNA, proteins, and cells) combined or integrated with microfabricated substrates. These devices are of interest for numerous applications, ranging from drug discovery, to environmental monitoring, to tissue engineering. Before a bio-microdevice can be fully developed, specific fabrication issues need to be addressed. One of the most important is the spatial immobilization of selected biomolecules in specific micro-areas of the device. Among the biomolecules of interest, the controlled immobilization of proteins to surfaces is particularly challenging due to the complexity of these macromolecules and their tendency to lose bioactivity during the immobilization step. The present Thesis reports on three novel micropatterning techniques for the spatial immobilization of proteins with bioactivity retention and improved read-out of the resulting micropatterns. The technologies developed are based on three different micropatterning approaches, namely 1) direct-writing UV laser microablation (proLAB), 2) a novel microcontact printing method (µCPTA) and 3) a replica molding method combined with bead self-assembly (BeadMicroArray). The first two technologies, proLAB and µCPTA, are an implementation of existing techniques (laser ablation and µCP, respectively), whereas the third, i.e., the BeadMicroArray, is a totally new technique and type of patterning platform.

‘ProLAB’ is a technology that uses a micro-dissection tool equipped with a UV laser (the LaserScissors®) for ablating a substrate made of a layer of ablatable material, gold, deposited over a thin polymer layer. The latter layer is transparent to the laser but favours protein adsorption. In the present work microchannels were chosen as the structure of interest with the aim of arranging them in ‘bar-codes’, so to create an ‘information-addressable’ microarray. This platform was fabricated and its application to specific antigen binding demonstrated.

The second technique that was developed is a microstamping method which exploits the instability of a high-aspect ratio rubber stamp fabricated via soft-lithography. The technique is denominated microcontact printing trapping air (µCPTA) since the
collapsing of a rubber stamp made of an array of micro-pillars over a plane glass surface resulted in the formation of a large air gap around the entire array. The method can be successfully employed for printing micro-arrays of proteins, maintaining biological activity. The technique was compared with robotic spotting and found that microarrays obtained with the \( \mu \text{CPTA} \) method were more homogeneous and had a higher signal-to-noise ratio.

The third technique developed, the BeadMicroArray, introduces a totally new platform for the spatial addressable immobilization of proteins. It combines replica molding with microbead self-assembling, resulting in a platform where diagnostic beads are entrapped at the tip of micropillars arranged in a microarray format. The fabrication of the BeadMicroArray involves depositing functional microbeads in an array of V-shaped wells using spin coating. The deposition is totally random, and conditions were optimised to fill about half the array during spin coating. After replica molding, the resulting polymer mold contains pyramid-shaped posts with beads entrapped at the very tip of the post. Thanks to the fabrication mode involved, every BeadMicroArray fabricated contains a unique geometric code, therefore assigning a specific code to each microarray. In the present work it was demonstrated that the functionality of the beads after replica molding remains intact, and that proteins can be selectively immobilized on the beads, for instance via biorecognition. The platform showed a remarkable level of selectivity which, together with an efficient blocking towards protein non-specific adsorption, lead to a read-out characterized by a very good signal-to-noise. Also, after recognition, a code was clearly visible, therefore showing the encoding capacity of this unique microarray.
ACKNOWLEDGMENTS

The work reported in this Thesis was partially funded by the CRC for Microtechnology and Nanotechnology Victoria (NanoVic), to which I would like to express my gratitude for supporting my studies.

Working towards this PhD was a long journey, and I would like to thank my supervisors who have helped me made it to the end. In particular, I would like to thank Professor Dan V. Nicolau for his support, encouragement and for all his input in the project.

A big ‘thank you’ to all the people who have assisted my research during those years, in particular Associate Professor Elena Ivanova, for teaching me to work with proteins; Dr Michael Abdo from CSIRO (Melbourne), for assistance with experiments with the microarrayer; Dr. Hans Brinkies for doing the SEM imaging; Kristi Hanson, for her help with the LSCM and her friendship; Peter Livingston for doing the AFM imaging; and Dr Prashant Sawant for useful discussions on gold ablation. Thanks also to Geri Solana, Codrin Mocanasu and to the entire IRIS staff for their encouragement and support; and to Fiona O’Donnell for her help with library-related issues.

Settling easily in Australia and completing this PhD would not have been possible without the friendship of a very special group of people, especially after becoming a first-time mum during my second year of candidature. There are not enough words to thank my husband and friend, Andrea, for his love, encouragement and patience, and my mother, who has moved from the U.S to Australia to help me completing this PhD. Your emotional and practical support has been really important to me. Thank you also to all my Australian friends; to the staff at SWiCH (Monash University) for looking after my daughter with joy and commitment while I was working towards this PhD; and Gina Ford, a very special book author.

Finally, I would like to acknowledge the support received from my family and friends in my home country, Italy. In particular, my father, who might not have my passion for science, but who has given me tremendous support during those years. My last thanks go to Ilaria, my little sunshine girl, whose smile gave me the energy to finish this Doctorate.
DECLARATION

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university or college of advanced education, and to the best of my knowledge and belief, contains no material previously published or written by another person except where due reference has been made.

Luisa Filipponi

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LIST OF PUBLICATIONS

The following is a list of publications that report work which is presented in this Thesis.


A patent has been filed in relation to the novel method described in Chapter 6:


During my candidature I have also contributed to the following book chapters:


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<th>Description</th>
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<td>αCP</td>
<td>Affinity contact printing</td>
</tr>
<tr>
<td>AFM</td>
<td>Atomic force microscopy</td>
</tr>
<tr>
<td>ALAPP</td>
<td>Allylamine plasma polymer</td>
</tr>
<tr>
<td>BMA</td>
<td>BeadMicroArray</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CAP</td>
<td>Chemical amplification photoresist</td>
</tr>
<tr>
<td>CCD</td>
<td>Charge coupled device</td>
</tr>
<tr>
<td>CFM</td>
<td>Chemical force microscopy</td>
</tr>
<tr>
<td>DIC</td>
<td>Differential interference contrast</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DNQ</td>
<td>Diazonaphthoquinone</td>
</tr>
<tr>
<td>DPN&lt;sup&gt;TM&lt;/sup&gt;</td>
<td>Dip-pen nanolithography</td>
</tr>
<tr>
<td>EDC</td>
<td>1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride</td>
</tr>
<tr>
<td>EDX</td>
<td>Energy dispersive X-ray spectroscopy</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>GPTS</td>
<td>3-glycopropyl trimethoxysilane</td>
</tr>
<tr>
<td>HMDS</td>
<td>1,1,1,3,3,3-hexamethyldisilazane</td>
</tr>
<tr>
<td>HPLC</td>
<td>High-pressure liquid chromatography</td>
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<tr>
<td>IEP</td>
<td>Isoelectric point</td>
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<tr>
<td>IgG</td>
<td>Immunoglobulin</td>
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<tr>
<td>IS</td>
<td>Ionic strength</td>
</tr>
<tr>
<td>LF</td>
<td>Lateral force</td>
</tr>
<tr>
<td>LFM</td>
<td>Lateral force microscopy</td>
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<tr>
<td>LSCM</td>
<td>Laser scanning confocal microscopy</td>
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<tr>
<td>MAPLE DW</td>
<td>Matrix-assisted pulsed laser evaporation direct writing</td>
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<tr>
<td>MEMPAT</td>
<td>Membrane-based patterning</td>
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<tr>
<td>μCP</td>
<td>Microcontact printing</td>
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<td>μCPTA</td>
<td>Microcontact printing trapping air</td>
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<tr>
<td>μFN</td>
<td>Microfluidic network</td>
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<tr>
<td>μMIA</td>
<td>Micro mosaic immunoassay</td>
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<tr>
<td>μTAS</td>
<td>Micro total analysis system</td>
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<tr>
<td>MIMIC</td>
<td>Micromolding in capillaries</td>
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<tr>
<td>MMA</td>
<td>Methylene methacrylate</td>
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<tr>
<td>nCP</td>
<td>Nanocontact printing</td>
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<tr>
<td>NHS</td>
<td>N-hydroxysuccinimide</td>
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<tr>
<td>NVOC</td>
<td>Nitroveratryloxycarbony</td>
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<tr>
<td>PBS</td>
<td>Phosphate buffer saline</td>
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<tr>
<td>PDMS</td>
<td>Poly(dimethylsiloxane)</td>
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<tr>
<td>PEB</td>
<td>Post-exposure bake</td>
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<tr>
<td>Acronym</td>
<td>Full Form</td>
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<tr>
<td>PEG</td>
<td>Poly(ethylene glycol)</td>
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<td>PEO</td>
<td>Poly(ethylene oxide)</td>
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<tr>
<td>PES</td>
<td>Poly(ether sulfone)</td>
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<tr>
<td>PET</td>
<td>Poly(ethylene terephthalate)</td>
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<tr>
<td>PGMEA</td>
<td>Propylene glycol methyl ether acetate</td>
</tr>
<tr>
<td>PI</td>
<td>Poly(imide)</td>
</tr>
<tr>
<td>PMMA</td>
<td>Poly(methyl methacrylate)</td>
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<td>ProLAB</td>
<td>Protein immobilization in laser ablated bilayers</td>
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<tr>
<td>PtBuMA</td>
<td>Poly(tert-butyl methacrylate)</td>
</tr>
<tr>
<td>PVA</td>
<td>Poly(vinyl alcohol)</td>
</tr>
<tr>
<td>QD</td>
<td>Quantum dot</td>
</tr>
<tr>
<td>ROI</td>
<td>Region of interest</td>
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<tr>
<td>S/N</td>
<td>Signal-to-noise</td>
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<tr>
<td>SAM</td>
<td>Self-assembled monolayer</td>
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<tr>
<td>SEM</td>
<td>Scanning electron microscopy</td>
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<tr>
<td>SNOM</td>
<td>Scanning near field optical microscopy</td>
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<tr>
<td>SPL</td>
<td>Scanning probe lithography</td>
</tr>
<tr>
<td>SPM</td>
<td>Scanning probe microscopy</td>
</tr>
<tr>
<td>SSCM</td>
<td>Stage scanning confocal microscopy</td>
</tr>
<tr>
<td>STM</td>
<td>Scanning tunnelling microscopy</td>
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<tr>
<td>ToF-SIMS</td>
<td>Time-of-flight secondary ion mass spectroscopy</td>
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<tr>
<td>UV</td>
<td>Ultra violet</td>
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<td>XPS</td>
<td>X-ray photoelectron spectroscopy</td>
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This Thesis is dedicated to my two-year old daughter Ilaria.
1 INTRODUCTION AND RATIONALE

Bio-microdevices are miniaturised devices based on biologically derived components (e.g., DNA, proteins, and cells) combined or integrated with microfabricated substrates. Methods that had been originally developed for the microelectronics industry, such as photolithography, and for the fabrication of microelectromechanical systems, such as silicon micromachining, were the first to be adapted to the fabrication of bio-microdevices. Research in this field started in the late 1970s, and resulted in the development of the first miniaturized gas chromatography system on a silicon chip (1). It was in the 1990s, though, that the scientific community recognized the tremendous potentials of microfabrication in biosciences, when Fodor and colleagues at Affimax (Stanford University) reported using photolithography for the fabrication of peptide and DNA arrays (2), (3). The technique was later optimized and a commercial product developed, the GeneChip® (Affymetrix), now employed in the next phase of the sequencing of the Human Genome (4).

However, the last ten years have witnessed a truly exponential growth in the research for the development of miniaturised bio-devices. Today, this field of research represents one of the most important sectors of the biotechnology and biomedical industry, with applications ranging from drug discovery, to environmental monitoring, to tissue engineering. Research in this area has been motivated by the numerous advantages conferred by miniaturization, such as the reduction of device size, use of smaller volumes and consequent reduction of waste, high-throughput and batch processing (5).

Before a bio-microdevice can be fully developed, many specific fabrication and biointeraction challenges must be addressed. Compared to silicon-based electronic devices, the challenges for the fabrication of bio-microdevices are much more diverse because of the large variety of biomaterials, fluids, and chemicals involved. Among these challenges is the spatial immobilization and the retention of bioactivity of selected biomolecules in specific micro-areas of the device. This is the central issue addressed in this Thesis.
1.1 Spatially Controlled Protein Immobilization

The immobilization of proteins in selected areas of a surface, including micro-areas (or ‘micropatterns’) of any shape, is defined ‘spatial immobilization’. The technology is often called ‘protein patterning’. When the identity of a spatially immobilized protein is defined by its position (X, Y coordinates) on the surface, the method is called positional immobilization (6). The term ‘array’ is often associated to this type of platform. Because the position of each pattern is known, positional arrays are also defined ‘spatially addressable’.

There are two major strategies for the immobilization of proteins in a spatially addressable micropattern. First, one can deposit locally the target protein from solution mechanically on the surface in the form of small droplets. This direct-writing approach relies on engineering methods such as robotic spotting (7). Robotic spotting allows fast printing over large surfaces using picoliters quantities of protein solutions. Unfortunately, the method often leads to uneven adsorption of the protein within the spotted area, which results from a combination of effects, e.g., the temperature of the substrate, solution and printing environment, the viscosity of the solution, the type of protein spotted, and the type of substrate, just to mention few. The main problem is the evaporation of the protein droplet, which generates uneven concentration throughout the spot, creating spots having a typical ‘donut shape’ (8). At times, bioactivity of the spotted proteins is lost due to those effects.

The second approach to protein patterning is to alter the properties of a micro-area, for instance with the use of light, creating a different chemical functionality locally. Subsequently, this ‘island’ is used for the selective immobilization of the target protein. This is a surface micropatterning approach, where the surface properties are tailored to selectively immobilize the proteins, but also to resist non-specific binding of other species in solution. Surface micropatterning is the method employed in the work reported in this Thesis.

Those two strategies have in common the use of substrates that have physiochemical characteristics which are favourable for the immobilization of the desired target, but differ for the lateral resolution of the smallest pattern they can fabricate. Whereas surface micropatterning methods can easily generate patterns down to 1 µm and lower,
the typical spot size obtained with a mechanical spotting system (e.g., microarrayer) is around 150 µm. A higher micropatterning resolution is advantageous for various reasons. For instance, it translates in the ability to fabricate smaller platforms (e.g., microarrays) with increased number of tests (i.e., types of biomolecules or cells) and increased number of tested biomolecules on the same unit area (i.e., density). The consequence is a reduction of processing costs associated with the use of less volume of analytes. Also, it increases the efficiency and reliability of the device, due to the increased number of tests that can be performed for each biomolecule on the same micro-patterned area (i.e., repetitions) (9). Another advantage of surface micropatterning is that it allows fabricating patterns with dimensions comparable with that of the biosystem of interest (e.g., proteins, bacteria, cells). Being able to tailor pattern size and properties according to the biosystem under investigation open numerous opportunities for manipulating biomolecule and cell inside miniaturized devices. At the same time, though, it triggers the challenge of avoiding unwanted interactions between the material and the biosystem being studied.

Among the numerous biomolecules involved in the development of a bio-microdevice, the controlled immobilization of proteins to surfaces is particularly challenging. In contrast with DNA, for which suitable immobilization technologies have been developed and DNA microchips commercialized, technology allowing uniform and global attachment of a wide variety of proteins is not currently available. This lag in technology arises from the fundamental structural difference between DNAs and proteins, the latter being a complex three-dimensional biomolecule with many attachment sites, which can loose its bioactivity if its structure is disrupted during the immobilization process. Also, proteins tend to attach non-specifically to many surfaces; therefore, in protein micropatterning technology, surfaces must be designed and prepared to optimize the immobilization of specific targets, but also to resist non-specific binding of other species in solution.

This need has triggered an extensive research in the development of suitable methods for the spatial immobilization of proteins over surfaces. The main target is the development of protein microarrays for proteome applications, i.e., the large-scale study of proteins, in terms of structure and function (6). Branches of proteomics include protein separation, identification, sequencing and interaction. Protein patterning finds
applications also in the field of biosensors, which exploit the sensitivity and selectively of some proteins like antibodies (10). It is also a key element in the development of total microanalysis system (μTAS), or ‘lab-on-a-chip’ (11), (5), for instance for blood separation and analysis, often microfabricated out of polymeric materials, over which proteins have a tendency to spontaneously adsorb. In the latter case, the μTAS needs to be engineered to prevent protein adsorption. Finally, protein patterning is critical in all cell-based microdevices, since in most cases proteins mediate the attachment of cells to surfaces. These devices include cell-based biosensors (12), which exploit the natural sensing capability of cells for the detecting specific metabolites and toxins; cell-based microarrays for combinatorial screening, for example for the screening of new drugs (13) and for fundamental biological studies (e.g., cell-cell communication) (14); patterned surfaces for controlling cellular growth (15), with application in tissue engineering (16), neuron regeneration studies (17-19), and brain modelling with artificially cultured neurons (20).

1.2 AIM AND SCOPE

The aim of the research presented in this Thesis is the development of micropatterning techniques for the spatial immobilization of proteins with bioactivity retention and improved read-out of the resulting micropattern. Microfabrication is employed as the patterning tool, and among the numerous methods available, preference is given to those which are easily accessible to biological laboratories. The performance of these new techniques is assessed by their selectively, i.e., capability of recognizing specifically a target protein, and by their read-out, in terms of signal-to-noise.

1.3 THESIS STRUCTURE

Protein immobilization on surfaces is a very wide field of research, whose fundamentals are summarized at the beginning of Chapter 2. The Chapter continues with a short overview of microfabrication methods involved in surface micropatterning, and then reviews the literature on protein patterning, dividing it in physiochemical, direct and topographical patterning methods. The Chapter discusses their advantages and
limitations, and draws conclusions that justify the choice of the methods employed in the work reported later.

Chapter 3 describes the experimental methods used in the work reported in this Thesis, which include fabrication methods (direct laser writing and soft-lithography), and characterization methods (fluorescence microscopy, atomic force microscopy and scanning electron microscopy). Some general considerations on the protein immobilization conditions that were employed in the experimental work are also reported.

The three Chapters that follow (Chapter 4, Chapter 5 and Chapter 6) report the new micropatterning techniques that were developed in this work, and their application to protein spatial immobilization. The techniques are assessed in terms of bioactivity retention of the patterned proteins, bio-specificity and quality of read-out.

The conclusions of this work are drawn in Chapter 7, where limits and improvements to the techniques are also suggested.
2 SPATIAL IMMOBILIZATION OF PROTEINS ON SURFACES

2.1 INTRODUCTION

The present Chapter reviews the surface micropatterning methods for the spatial immobilization of proteins (i.e., protein patterning). Protein patterning is a physically defined form of protein immobilization; therefore the Chapter starts with a brief overview of the fundamentals of the immobilization of proteins to surfaces, describing the types of immobilization that can be employed, namely physical adsorption, covalent binding and biorecognition. This first part is a summary of fundamental concepts that we have recently reviewed (21).

Most protein patterning methods employ a microlithographic-approach at same stage of the process, therefore the Chapter continues with a short overview of this technology, followed by a literature review on the spatial immobilization of protein to surface. Strategies to pattern proteins on planar surfaces are divided in (1) methods that involve physiochemical modification of surfaces; (2) methods that allow direct patterning and (3) methods that involve topographical modification of surfaces. Physiochemical patterning refers to the use of substrates with planar patterns to modulate physical as well as chemical interactions between the protein and the surface. Within physiochemical methods, a separate section is dedicated to biochemical patterning, i.e., the fabrication of patterns of specific biomolecules to attach proteins based on biomolecular recognition. Direct patterning refers to methods that deliver proteins to surface with the use stamps, stencils or ‘nano-pens’. Topographical patterning refers to the fabrication of features on a substrate (e.g., laser ablated grooves) to restrict the location of proteins within the contoured environment.
2.2 TYPES OF IMMOBILIZATION

2.2.1 Physical adsorption

The simplest type of protein immobilization to a surface is through physical adsorption, where mutual attraction between the solid surface and the protein determines the coverage of the surface. This process relies on non-covalent interactions (mainly electrostatic, van der Waals, and dehydration of hydrophobic interfaces) and is of a purely physical nature; therefore it displays varying levels of reversibility. Hydrophobic interactions are often stronger and less reversible than electrostatic interactions but can be associated with conformational changes in molecular structure, as the hydrophobic interior of the biomolecule ‘unfolds’ to position itself against the hydrophobic interface. At times, this can lead to denaturation of the biomolecule and loss of its biological activity.

2.2.1.1 Surfaces that favour protein adsorption

Surfaces that are commonly used for immobilizing proteins through physical adsorption include hydrophobic polymers, such as poly(styrene), poly(methylmethacrylate), and poly(dimethylsiloxane), cationic surfaces like polylysine-coated glass slides, glass or silicon substrates chemically modified to acquire hydrophobic surface properties (e.g., by treatment with a trimethoxysilane), and self-assembled monolayers (SAMs) of alkanethiolates on gold (22).

2.2.1.2 Surfaces that resist protein adsorption

Protein adsorption is dramatically reduced on hydrophilic substrates, such as glass, hydrophilic polymers, such as agarose, poly(vinyl alcohol, PVA), poly(ethylene oxide, PEO), also known as poly(ethylene glycol, PEG), and organic monolayers functionalized with specific chemical groups such as oligo(ethylene oxide) (23) and tripropylene sulfoxide (24). Oligo(ethylene oxide)-terminated SAM is abbreviated EGₙ-SAM or EGₙOH-SAM. Research done by Prime and co-workers (23) has shown that longer EGₙ chains (3<n<6) resist adsorption more effectively than shorter chains. For this reason, EG₆OH-SAM is normally employed for preventing protein adsorption.
Surfaces treated with the protein albumin also inhibit protein adsorption. For instance, bovine serum albumin (BSA) is a commonly used blocking agent in antibody-based assays, such as ELISA (25). In the work reported in this Thesis, surfaces were often treated with BSA to prevent protein adsorption.

2.2.2 Covalent binding

This is a more stable means of immobilization, based on the formation of a chemical bond between the solid surface and the protein (21). Proteins present a variety of functional groups, including amino-, carboxyl-, hydroxy- and thiol-, which can readily be used for covalent binding to surfaces with complementary chemical groups. However, due to the relative lability of proteins care is required to avoid chemically-induced protein denaturation during the attachment process. There are many strategies for cross-linking of available functional groups, most of which make use of specialized cross-linkers designed for both attachment and physical separation of the protein from the surface, thereby allowing for more of the protein functional domain to be exposed to the solvent.

2.2.2.1 Substrates for covalent binding

Among the most commonly used substrates are amino- and carboxyl-modified surfaces, which immobilize proteins through their amino groups, with the formation of an amide linkage (22). With amino-terminated surfaces, glutaraldehyde or N-hydroxysuccinimide (NHS) are used for cross-linking, whereas with carboxyl-terminated surfaces a carbodiimide (e.g. 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride, EDC or EDAC) is used to mediate the formation of the amide linkage with the protein (26). Other surfaces used for protein covalent attachment include aldehyde- and isocyanate-modified substrates, chlorinated glass slides (to react with hydroxy groups in the protein), and maleimide-modified substrates (to react with thiol groups in the protein) (22), (26).

2.2.3 Biorecognition

Biorecognition is an immobilization technique that exploits the natural complementarities of some biomolecules. These biomolecules are therefore high-affinity ligand pairs and provide stable immobilization similar in strength to covalent
binding. Biotin-avidin and antigen-antibody pairs are two widely utilized high-affinity ligand pairs, and both were used in the work reported in this Thesis.

### 2.2.3.1 Biotin-avidin

Avidin is a glycoprotein found in egg whites that contains four identical subunits, each having one binding site for biotin (or vitamin H) \((27), (28)\). The avidin-biotin interaction is among the strongest non-covalent affinities known: harsh conditions (e.g., 6-8 M guanidine at pH 1.5) are required for inducing complete dissociation. The only limitation of avidin is its tendency to bind non-specifically to components other than biotin, which is due to its high pI (about 10) and carbohydrate content. Streptavidin is another biotin binding protein isolated from *Streptomyces avidinii* that is often used to overcome some non-specificities of avidin. Similarly to avidin, streptavidin contains four subunits, each with a single biotin binding site \((27)\). The primary structure of streptavidin is considerably different than that of avidin, which is reflected in a much lower isoelectric point (pI 5-6). Both proteins are very stable and can tolerate a wide range of buffer conditions, pH values and chemical modifications. Both proteins can be conjugated to other proteins or labelled with various detection agents without loss of binding activity \((27)\).

### 2.2.3.2 Antigen-antibody

IgGs are a class of immunoglobulines, a special group of glycoproteins that recognizes a specific antigen unique to their target. IgGs have a characteristic “Y”-shape structure that contains two identical heavy chains and two identical light chains linked via disulfide bonds \((29)\) (Figure 2.1). The arms of the Y (the Fab regions) contain the antigen-binding sites, whereas the vertical line is called the Fc region. When used as a sensing element over a solid support, it is critical that the IgG molecule is immobilized in an oriented manner, i.e., with the antigen-binding sites exposed to the solution and available for recognition.
Orientation of immobilized IgGs is increased using protein A, a protein that binds to the Fc domain of IgG. Protein A has four IgG-binding sites, thus regardless of the orientation of protein A on a surface, two or more IgG-binding sites remain exposed to solution and available for coupling. Protein G, like protein A, binds to the Fc domain of IgG (30). Protein G binds more IgG isoforms than protein A and does not cross-react with other immunoglobulines like IgM, thus is more selective and more universal. Interestingly, though, a study that compared the two proteins showed that antibodies oriented with protein A had a greater antigen-binding enhancement compared with that of non-oriented samples than those oriented with protein G (31). This result is anomalous because protein G immobilizes more IgG per square centimetre than protein A. Lu and co-workers explained this results as probably due to the orientation of IgG on protein G monolayers, which was not optimal for antigen binding (31). This demonstrates that the ability to orient antibodies with their antigen-binding site exposed to solution is not necessarily sufficient to maximize activity. Also, steric interactions between neighbouring proteins may decrease binding activity.
2.3 **MICROPATTERNING METHODS**

A protein patterning method may adopt one of these strategies: (a) promote selective protein attachment over a surface with patterns of localized physical, chemical or biochemical functionality (physiochemical patterning); (b) provide physical means for depositing proteins, such as a stamp, or a barrier to limit areas of adsorption (direct patterning); and (c) use a surface with localized topography, to limit protein attachment inside the micro-grooves.

All these strategies have in common the use of a micron-sized pattern made with a microfabrication method. Microfabrication technology includes numerous microprocessing methods originally developed for the microelectronics industry. Some of these methods (e.g., photolithography) have been applied to the fabrication of micropatterns for the spatial immobilization of proteins, while others have been developed specifically for this application (e.g., soft-lithography).

A complete review of microfabrication methods is beyond the scope of this Thesis; therefore this Section provides a short description of the micropatterning methods which are relevant to protein patterning. Later in the Chapter, the application of these microfabrication methods for the spatial immobilization of proteins in micron-sized patterns will be reviewed.

### 2.3.1 Lithography

Lithography includes a series of microfabrication techniques that share the principle of transferring an image from a mask to a receiving substrate (32). A typical lithographic process consists of three successive steps: (i) coating a substrate (Si wafer or glass) with a sensitive polymer layer (resist), (ii) exposing the resist with light, electrons or ion beams, (iii) developing the resist image with a suitable chemical (developer), which reveals a positive or negative image on the substrate depending on the type of resist used (i.e., positive tone or negative tone resist). In conventional microfabrication, the next step after lithography is the pattern transfer from the resist to the underlying substrate. This is achieved with a number of transfer techniques, such as chemical etching and dry plasma etching.
Lithographic techniques can be broadly divided in two main groups:

1. Methods that use a *physical mask*, where the resist is irradiated through the mask which is in contact or in proximity with the resist surface. These methods are collectively called *mask lithography*, among which photolithography often employed in protein patterning.

2. Methods that use *software mask*. Here, a scanning beam irradiates the surface of the resist sequentially, point by point, though a computer-controlled program where the mask pattern is defined. These methods are collectively called *scanning lithography*.

The main difference between mask and scanning lithography is speed: whereas mask lithography is a parallel, fast technique, scanning lithography is a slow, serial technique. Another important difference of these two sets of techniques is resolution which, in general terms, is higher for scanning methods. The price for higher resolution, though, is the use of more energetic radiation sources which translates in expensive equipment.

Many protein patterning methods utilize a lithographic-based approach at some point in the process. In conventional microfabrication, the next step after lithography (being this mask or scanning lithography) is the pattern transfer from the resist to the underlying substrate (Figure 2.2 (a)). When applied to biopatterning, the method is applied differently. For instance, photolithography is used for the generation of surface patterns with localized chemical functionalities for binding selectively a biomolecule (e.g., protein) of interest (Figure 2.2 (b)). Radiation-sensitive materials used in this case need to be biocompatible with the biosystem they are interfacing with and include materials specifically synthesized as well as selected conventional photoresists, such as positive tone resists and chemical amplification resists. The use of photolithography for biopatterning will be further discussed later in this Chapter.
2.3.1.1 Photolithography

Photolithography uses light (UV, deep-UV, extreme-UV or X-ray) to expose a layer of radiation-sensitive polymer (photoresist) through a mask (32). The mask is a nearly optically flat glass (or quartz, depending on the light used) plate which contains the desired pattern: opaque areas (the pattern, made of an absorber metal) on a UV-transparent background. The image on the mask can be either replicated as it is, placing the mask in physical contact with the resist (contact mode photolithography) or reduced, usually by a factor of 5 or 10, and projected to the resist layer through an optical system (projection mode photolithography).

The resolution of contact mode lithography is typically 0.5-0.8 µm when UV light (360-460 nm) is used (32). Higher resolutions cannot be achieved due to the inability to reduce the gap between the mask and the flat substrate below ~1 µm, even when elaborate vacuum systems are used to hold the two parts together. To produce patterns with higher resolution, projection photolithography or ‘next-generation photolithography’ techniques (i.e., extreme UV and X-ray photolithography) need to be employed (33), (32). These technologies use very expensive equipment and therefore their use is limited to selected applications (such as photomask fabrication).
2.3.1.2 Scanning lithography

A UV laser can be used instead of a broadband UV source for the direct exposure of a photosensitive surface, in which case the technology is called laser beam lithography. When high energy lasers are used the interaction of the laser with the polymer breaks the backbone of the molecule in a process defined laser ablation (34), (35). The ablated region contains the decomposed polymer, which is generally removed through a flow of pure nitrogen. The result is the formation of topographical features, e.g., microgrooves. Feature sizes in the order of microns can be easily achieved.

Energetic particles such as electrons and ions can be used to pattern appropriate resist films leading to features with nanometre resolution. When using electrons, the technology is called electron beam lithography (e-beam), whereas when using ions the technology is called focused ion beam lithography (32). Finally, a recently established technology uses nanometre scanning probes for patterning resist films and is therefore referred to as scanning probe lithography (SPL). This technology has been extended to the deposition of nano-quantity of material (Dip-pen nanolithography, (DPN™)) (36).

2.3.2 Soft-lithography

Soft-lithography is a name for a number of techniques that fabricate and use a soft mold prepared by casting a liquid polymer precursor against a rigid master. These microfabrication techniques have been specifically developed by Whitesides’ group (Harvard University) to be used for biological applications (37), (38).

The resolution of soft-lithography is mainly determined by van der Waals interactions, by wetting, by kinetic factors such as filling the capillaries on the surface of the master, but not by optical diffraction (33). This is an important advantage over ‘conventional’ lithographic techniques. The master is normally fabricated via a conventional lithographic method.

Various polymers (e.g., polyurethanes, epoxides, and polyimides) can be used for molding and provide a range of possible materials and properties of the final mold. Most commonly, though, the elastomer poly(dimethylsiloxane) (PDMS) is used (39). The reason for this lies in the numerous advantageous properties of this material, especially when used with biological materials. PDMS has a very low glass transition
temperature (-130°) and surface tension (21 mN/m). The material is hydrophobic but can be rendered hydrophilic by treatment with oxygen plasma, corona discharges or exposure to deep UV in the presence of oxygen¹ (40). The polymer is also biocompatible and optically transparent down to 300 nm and thermally stable (below 150 °C), resistant to many chemicals and pH environments, non toxic, gas permeable (in particular N₂ and O₂ permeable) but water impermeable, durable and reusable (a PDMS stamp can be repeatedly used without loosing its resolution up to 50 times). Also, thanks to its elastomeric properties, a PDMS stamp can be sealed reversibly to both planar and non-planar surfaces. All these physiochemical properties make PDMS a polymer well suited for interfacing with biological materials.

A PDMS mold is fabricated by pouring its liquid precursor over a lithographically-made master (e.g., a photoresist or silicon master), cured to induce cross-linking, and then peeled off from it. The stamp can then be used for either printing a desired material (the ‘ink’) from the stamp to a suitable surface (microcontact printing, µCP) or, when in contact with a flat or curved surface, to define physical constrains where a liquid can be confined. The latter includes two techniques: micromolding in capillaries (MIMIC), where microchannels formed by the contact of the stamp with a substrate are used to deliver a fluid to restricted areas; membrane-based patterning (MEMPAT), where an elastomeric membrane with holes, in contact with a substrate, is used to limit the deposition of a desired material within the physical constraints of the holes. Soft lithography applied to protein patterning is schematized in Figure 2.3.

¹ PDMS recovers its original hydrophobicity if left in air after the oxidation treatment. To prevent this from happening, oxidised PDMS needs to be kept in contact with water.
Figure 2.3 Scheme of soft lithography and its application to protein patterning.
2.4 PROTEIN PHYSIOCHEMICAL PATTERNING

Physiochemical patterning refers to the use of substrates that have patterns with defined physical (e.g., electrical), chemical or biochemical properties. Early works in the field have relied on substrates with poorly characterized chemical properties, such as plasma irradiated polymers. Later, substrates with homogenous properties were chosen, such as ultra flat surfaces of metals, polymers or organic monolayers. The following Section reviews those works, which have been grouped based on the use of (i) self-assembled monolayers; (ii) photolithographic patterning; (iii) biochemical patterning; and (iv) direct patterning. This Section includes also a new direction in protein patterning, i.e., the use of patterns of microbeads.

2.4.1 Patterning with Self-assembled monolayers (SAMs)

Some organic molecules, when exposed from solution or vapour to a suitable substrate, self-assemble to produce homogeneous, densely packed layers of monomolecular thickness. These organic molecules have long chains with two different end-groups. The monolayer is formed when one of the two end groups of the organic molecule reacts with a particular surface forming a chemical bond. The surface properties of the substrate are then defined by the exposed functional groups of the monolayer. For instance, alkyl-silane or alkane thiol molecules exposed to a silica or metal surface assemble into organized layers. SAM-forming materials may be *physisorbed layers*, such as Langmuir-Blodgett films, or *chemisorbed layers*, such as organosilanes bonded to silica or organothiols bonded to gold (41). Both types of SAM films inherently offer a high degree of control in the vertical direction at molecular level, but chemisorbed films are more versatile and stable, because of the stronger covalent binding between the film precursor and the surface. Films of mixed SAMs with tailored surface properties can be fabricated by mixing two (or more) precursor molecules and photosensitive SAM layers can be produced by molecularly engineering the precursor to have a photoreactive specie.
2.4.1.1  Two-component silane patterns

Conventional photoresist technology, originally developed for patterning metals to fabricate microcircuits, is one of the first technologies that were adapted to generate chemisorbed SAMs patterns for protein immobilization. In conventional photoresist technology a substrate is coated with a photoresist, exposed and developed to generate a pattern. Metal is then deposit over the substrate. Treatment with an organic solvent (e.g. acetone) dissolves the unexposed resist leaving the desired metal pattern (see Figure 2.2 (a)). In the biomolecule-patterning approach, instead of depositing metals on selected regions of the exposed substrate, chemical linkers with different pendant groups are used to create a heterogeneous monolayer.  *Silane-coupling chemistry* has been the preferred strategy to attach proteins to silica or metal surfaces, since silane-coupling agents can withstand the harsh solvent systems required to remove the photoresist (42). The substrate (e.g. silica) is spun cast with a photoresist and exposed to UV light through a mask. After development, a first silane, usually hydrophilic, is bound to the exposed regions. Removal of the remaining photoresist exposes the rest of the substrate, which is then incubated with a second, hydrophobic silane, resulting in a mixed monolayer. For instance, patterns of alternated aminosilanes and methyl-terminated alkylsilane SAM have been used for the spatial immobilization of proteins and some synthetic peptides (43). The biomolecules were immobilized covalently to the amino-terminated areas of the pattern, using glutaraldehyde as the cross-linker.

2.4.1.2  Patterns of SAMs on gold

Whitesides and his co-workers from Harvard University have introduced the use of mixed SAMs of alkanethiols on gold surface to control protein adhesion. SAMs of alkanethiol on gold are formed when a gold surface is exposed to a solution or to the vapour of an alkanethiol. The sulphur atoms of the alkanethiols coordinate with the gold surface, while the alkyl chains are closely pack and tilted by 30 degrees form the surface normal (44). The terminal end-group of a ω-substituted alkanethiol determines the surface properties of the monolayer.
Different methods, both lithographic and non-lithographic, have been reported for patterning SAMs of alkanethiols on gold. One of the most popular is microcontact printing (µCP) introduced by the above-mentioned research group. In this approach, the stamp is first ‘inked’ with a thiol-terminated alkane, printed on a gold surface and, subsequently, the surface incubated with a second alkanethiol which self-assembles onto the rest of the surface that is left clean by the stamp. Lopez and colleagues reported the use this method to generate a pattern of alternated methyl-terminated SAM and EG₆OH- SAM (45). Immersion of the patterned SAMs in a solution of protein (such as fibronectin, fibrinogen, pyruvate kinase, streptavidin and immunoglobulins) resulted in adsorption of the proteins on the methyl-terminated regions. The same method was also used to create a protein pattern to control cell adhesion (46), with control at the single-cell level.

Another method to fabricate patterns of SAMs on gold is to create first the gold micropattern and then to assemble the monolayer over this preformed pattern. For instance, Tender and co-workers described the use of laser ablation to created gold micropatterns on a glass substrate. Alkanethiols were subsequently assembled on the gold pattern and used for protein immobilization (47).

2.4.1.3 SAMs nanografting

A recently established technique uses a SAM monolayer as a resist surface, which is then removed in selective areas by means of the probe tip of an Atomic Force Microscope (AFM), which acts as an extremely sharp stylus to literally scrape away a swath of molecules. The technique is called Scanning Probe Lithography (SPL) or ‘Nanografting’. The freshly patterned surface is flooded with a patterning molecule which selectively binds to the exposed areas. Variations on this approach have been developed in the laboratory of Liu and others as relatively facile methods for fabrication of SAM nanopatterns, and then used to selectively bind proteins (48), (49). The AFM tip is used for nanografting a SAM substrate (CH₃-terminated SAM) and a second SAM is assembled in the exposed regions (COOH-terminated SAM). The nanopattern is then exposed to a solution of protein (lysozyme), which selectively adsorbs to the COOH-
terminated region. Alternatively, by assembling a SAM with a different functionality (e.g., aldheide), covalent attachment is obtained. The method allows fabricating patterns as small as 10 nm (49).

2.4.1.4 Patterning using SAMs: discussion

The main advantage of SAM patterns is the ability to control the nature and the density of their functional end-groups, which makes SAMs surfaces excellent model surfaces for protein adsorption studies (50).

The quality of the assembled monolayer is highly dependent on the nature of the substrate used. In particular the assembling of alkanethiols on gold requires an ultra-flat gold-coated surface with minimum roughness. This gold substrate is biocompatible; therefore SAMs on gold can be used for interfacing with biological systems, such as protein and cells. SAMs of alkanethiol on gold, though, are instable and desorb when exposed to temperature above 70 °C, when exposed to UV light in the presence of oxygen, or when exposed to atmospheric ozone (44). Therefore, samples need to be protected from intense light and temperature prior and during their use. SAMs instability represents a limitation for their use in commercial applications such as microarrays and biosensors.

2.4.2 Photolithographic patterning

Photolithographic techniques for immobilizing proteins use light (mostly UV or deep UV) to either activate or deactivate photosensitive chemical species, which are then used to bind the target. Areas with localized reactivity are created by selectively irradiating a photosensitive surface, e.g. through a mask. This lives a surface where proteins can be immobilized based on random adsorption or covalent attachment. These methods, thus, combine photochemistry with photolithography.

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2 This result appears to contradict the knowledge that proteins adsorb preferentially on hydrophobic surfaces (here, the CH$_3$-terminated SAM). At neutral pH, though, lysozyme is positively charged (pI 11.1), while the COOH-terminated SAM is partially negatively charged; thus electrostatic attraction is likely to be the driving force of the selective attachment of lysozyme (49).
In photolithographic patterning two parameters are essential: (1) the flatness of the substrate and (2) the contact between the mask and the surface. The flatness of the substrate is optimized by depositing a very thin film of photosensitive material over a clean slide (made of glass or silicon oxide) using a spin coater, which allows creating homogeneous films with a thickness dependent on the viscosity of the starting solution. Contact between the mask and the substrate is controlled using an exposure aligner where the two parts are held together under vacuum to minimize the air gap that is formed between the two parts. The photosensitive film is then baked to remove the solvent and then lithographically exposed (most of the times in contact-mode lithography, i.e., through a mask). The exposure tool generally is a broadband UV mercury lamp. The whole process is conducted in a clean room facility, which is also equipped with special lights to prevent the unwanted exposure of the substrate to the UV component of natural light.

The literature on photolithographic protein and peptide patterning can be divided in (1) works that use microlithographic-like procedures and (2) works that use microlithographic-like materials, i.e., photoresists. In both cases, a photosensitive surface is used, but in the first case it is made of a purposely synthesized compound modified to have a photosensitive group (such as arylazide-, nitrobenzyl-, diazirine- and benzophenone-functionalized compounds), whereas in the second case the material is a commercial photoresist, i.e., photosensitive polymer originally designed for the microelectronic industry.

2.4.2.1 Patterning via microlithographic-like procedures

Microlithographic procedures have been adapted to immobilize biomolecules on surfaces. One example has been already discussed, i.e. the formation of silane patterns using conventional microlithography. In that approach, though, the process is used only as a microfabrication tool, i.e. the photosensitive surface is exposed and developed to leave a pattern where silanes can be covalently immobilized to the revealed glass surface. Therefore the photosensitive surface is employed for biomolecule patterning but not involved in the process. When this happens, the technology is generally referred to as ‘photochemical patterning’.

Scenarios for photochemical protein patterning can be classified as follows:
2.4.2.1.1 Photochemical fixation

A substrate is functionalised with a photochemical group, incubated with a protein solution and irradiated with a mask. Illumination “activates” the substrate in localized regions through the generation of radicals that can then bind the protein in solution.

Arylazide photochemistry is an example of this method. The arylazide group (-C₂H₅N₃) upon photolysis with UV light is converted into a reactive nitrene which can insert into C-H bonds. In 1995 Matsuda and co-workers introduced the chemical fixation of proteins on surfaces based on the photochemical reactivity of the arylazide group, derivatized either to a protein or on a polymer surface (51).

2.4.2.1.2 Photochemical cross-linking

A substrate is incubated with a cross-linking polymer and a protein solution. The cross-linking polymer contains several photochemical groups so when the solution-covered substrate is irradiated localized regions of the substrate are activated. The activated polymer binds to the substrate and to the protein, acting as a cross-linker between the two.

Example of this method is the diazirine photochemistry. The diazirine group (-CH₂N₂) adsorbs light at 350 nm and forms highly reactive carbenes that can easily insert into covalent bounds. This method was used to immobilize diazirine-coupled proteins to surfaces and to immobilize a synthetic oligopeptide to a surface to mediate cell adhesion (52).

The above-described methods have in common the use of UV light together with a protein solution. Although in these works the irradiation wavelength was selected to prevent protein damage (λ>350 nm), this was not always investigated. It is now quite unpopular to adopt this approach for protein immobilization and a sequential approach is often preferred, i.e., first modification of a sensitive surface with UV light, then immobilization of the desired protein in a subsequent step.
2.4.2.1.3 Caging chemistry

A substrate is functionalised with a ‘caged’ specie that can be released upon exposure. By attaching the caged moiety to groups that can bind proteins or amino acids discrete protein patterns can be produced by selectively removing the cage via photolithography.

Fodor and colleagues (2) pioneered the use of caging chemistry to create arrays of peptides. A substrate was firstly functionalised with nitroveratryloxy carbonyl (NVOC), a photo-removable protecting group that generates amino groups upon exposure. The freed amino groups were then used to synthesize a series of peptides in an array format. The use of a 50 µm checkerboard pattern within 1.6 cm² allowed the creation of an array of 1024 sequence pentapeptides which was then used to bind an antibody and to identify the key sequence of the amino acids responsible for antibody binding (2). This micropatterning technology is now known as the Affymetrix method and is the technology behind the commercial GeneChip®.

2.4.2.1.4 Photochemical synthesis

A material (e.g. polymer) with photoreactive groups is coated over a substrate and selectively irradiated through the mask. Exposure has the effect of generating new functionalities over selective areas of the surface (most commonly carboxyl groups, but also amino or hydroxy groups). The pattern is then used to adsorb proteins based on the different physical properties of the unexposed/unexposed regions (such as different wettability) or to covalently bind the protein.

Matsuda et al. used surfaces functionalized with the o-nitrobenzyl ester group which, upon UV exposure, is converted into the corresponding carboxyl group. Surface generated carboxyl groups were the coupled to the amino groups of a protein (53).

2.4.2.2 Patterning via microlithographic-like materials (photoresists)

Nicolau and colleagues (54) have introduced the use of two commercial microlithographic materials for patterning proteins: diazonaphthoquinone/novolak (DNQ), the common resist used in contact mode photolithography, and poly(tert-butyl)methacrylate (PtBMA), a common e-beam/deep UV sensitive polymeric material.
Recently, chemical amplification photoresists (CAP) have also been used to selectively bind proteins (55).

2.4.2.2.1 UV photoresist

Diazonaphthoquinone/novolak (DNQ/Novolak) photoresist is a well-known UV photoresist. Upon UV exposure, carboxyl groups are generated on the polymer surface from the DNQ component of the resist, with a consequent change in surface wettability. The formation of carboxylic groups in the exposed regions of the polymer induces an increase in hydrophilicity, which in turn allows the diffusion of the aqueous basic solution (developer) and selective dissolution of the exposed areas.

When applied to protein patterning, the change in surface chemistry and wettability can be exploited to control protein attachment. In theory, two linkage mechanisms can be used, namely direct linkage (spontaneous adsorption) and induced linkage (covalent binding). In the first case, hydrophobicity-induced protein attachment can be achieved exploiting the different chemical properties of the exposed and unexposed regions. In the second case, a cross-linker is used to mediate surface-protein interaction through the formation of a covalent bound between the carboxyl group of the exposed regions and the terminal amino group of the protein. The latter method was reported to allow good yield and contrast, whereas the hydrophobicity-induced protein attachment method was unable to induce effective patterning (54).

Although DNQ/Novolak resists have been successfully used for pattern proteins and cells (56), (57) its use has some limitations: those resists are suitable to fabricate patterns with above-micron resolution and they are inherently fluorescent, a characteristic which may pose a problem if the detection method is fluorescence-based. Nevertheless, DNQ/Novolak resists are still considered of interest in the field of protein patterning. In particular, the ability of creating gradients of carboxylic-rich surfaces, with concentration modulated by exposure energy, to study protein absorbance modulation has not yet been fully explored.
2.4.2.2 *E-beam/Deep UV photoresists*

Polymers designed for e-beam/deep UV lithography have some advantages compared to UV photoresists: they are fluorescent-free and are capable of high-resolution printing. Those characteristics make them attractive candidates for protein patterning. The feasibility of using common e-beam/deep UV resist for protein patterning was investigated by Nicolau and co-workers, who in particular studied the use of P(tBuMA-co-MMA), a copolymer of tert-butyl methacrylate (tBuMA) with methyl methacrylate (MMA) (58). Exposure of the polymer to e-beam radiation induces hydrophilization of the PtBuMA component, with formation of carboxylic groups on the exposed resist. This chemical modification is reflected in a change of surface hydrophobicity, the material being hydrophobic when unexposed and progressively more hydrophilic upon exposure. Protein patterning was studied using two strategies: (i) a hydrophobicity selective attachment method based on the differences of hydrophobicity of the exposed/unexposed regions (physically controlled mechanism) and (ii) a chemically selective attachment method using the linkage between the terminal amino group of the protein with the carboxylic groups generated on the exposed areas of the resist (chemically controlled mechanism). The first method could be used to print negative tone protein images whereas the second method could be used to print positive tone protein images.

2.4.2.3 *Patterning with photoactive SAMs*

Dulcey and co-workers introduced the use of deep UV exposure to directly modify SAMs surfaces (41). In this method the SAM is exposed through a mask and the exposed regions absorb the radiation and undergo a chemical modification. The newly formed end-groups can then be modified with a different functionality. This technique presents some advantages compared to the silane-coupling method previously discussed (Section 2.4.1.1), i.e., fewer processing steps, higher resolution (down to about 0.3 µm) and compatibility with a large number of substrates.

Another group examined the use of thiol-terminated SAMs to create patterns of proteins (59). After UV irradiation, the pendant thiol group is converted to sulfonate group, which *inhibits* protein adsorption. The surfaces of thiol-terminated SAMs were exposed through a mask and the unexposed regions, which retained the thiol group, used to
either promote protein adsorption, or for protein covalent binding to mediate cell attachment (59).

Finally, Delamarche has described a method that combines photochemistry with alkenethiol SAMs on gold to create protein (IgG) patterns (60). In this method an alkane thiol with an active ester group is firstly assembled onto a gold surface. The ester group is then reduced to amino group and used to link a cross-linker bearing a benzophenone group. This is a photosensitive group that has some advantages compared to the other sensitive groups such as arylazide and diazide that were previously discussed. It is more stable then those, inert under ambient light and is activated by near UV ($\lambda$>350 nm), thus avoiding lower wavelengths that can be protein-damaging. The photoactive surface is then covered with the protein solution and irradiated with mercury lamp coupled to a band pass filter (320<$\lambda$<550 nm) through a mask. Irradiation causes bi-radical formation at the ketyl centre of the photolabile group, which is followed by C-C bound formation after radical recombination. The bioactivity of the patterned protein was demonstrated through specific binding to the corresponding antibody.

### 2.4.2.4 Photolithographic patterning: discussion

The main advantage of photolithographic patterning is that it relies on a well-established chemistry. Also, the ability to control the exposure energy during the irradiation process allows fabrication of patterns with gradients of chemical functionalities. The main limitations of this technique are the need to use specialized equipment in a clean-room facility, the nature of substrates it can use, i.e., an ultra-flat surfaces capable of photochemistry, and the resolution it can reach, which is intrinsic to the photolithography technology, i.e., above 100 nm. The use of deep UV/e-beam photoresists allows increasing the resolution of the pattern, but it also implies using a high-energy emitting exposure tool (e.g., e-beam machine), which is considerably more expensive that a conventional UV mask aligner.

Methods that use purposely-synthesized compounds are versatile in regards to the photochemistry they allow but have the disadvantage of needing numerous processing steps. The use of commercial photoresists avoids this timely procedure but introduces the problem of the inherent fluorescence of these materials.
2.4.3 Biochemical patterning

Biochemical patterning is based on engineering surfaces in a way that the target adheres to it based on *biomolecular recognition*. Examples of biorecognition can be found in numerous works that were reviewed in the previous sections. In those works, though, biorecognition was used to prove the retained bioactivity of a patterned protein. In this section, biorecognition is used as a method to mediate protein patterning.

2.4.3.1 Patterning through the biotin-avidin high affinity system

The biospecificity and strength of the biotin-avidin noncovalent interaction make this system very useful for the micropatterning of biomolecules. To exploit this interaction, biotin is linked to the biomolecule of interest (e.g., protein) or to the surface using a biotinylation agent, and then used as a site for the specific recognition of an avidin-functionalised target (Figure 2.4 (a) and (b)). Also, avidin (or streptavidin) can be used as homobifunctional protein adapters, i.e., each protein molecule can simultaneously bind to a biotin-functionalized substrate and biotinylated molecules on the opposite face exposed to solution, as illustrated in Figure 2.4 (c).
Some researchers have combined the benefits of pholithography and caging chemistry with that of the avidin-biotin specific association and used *photoactive caged biotin* for the spatial immobilization of various macromolecules and proteins. The Affymax Research Institute was the first to report this approach (61), already mentioned in Section 2.4.2.1. Their method uses a photolabile, caged biotin analogue (functionalized with a nitrobenzyl group) covalently linked to a solid substrate. UV irradiation through a photolithographic mask yields freed biotin sites now available for streptavidin binding. Biotinylated macromolecules are then immobilized via a biotin-streptavidin-biotin bridge. Reichert and co-workers extended this approach to a step-and-repeat patterning of multiple antibodies (62),(63). Here, BSA was first derivatized with a photoactive biotin and adsorbed to a glass surface. From here, the method follows the Affymax protocol and biotinylated antibodies are attached in the final step in a serial manner. Although the absorption of BSA to glass is quite robust, the authors reported
that denaturants could still disrupt this interaction. Therefore, covalent binding of the
caged-biotin compound is preferable.

A recent work (64) reports a complimentary method to micropattern proteins onto
SAMs on gold with a photolabile caged biotin derivate, a method denominated by the
authors ‘light-activated affinity micropatterning of proteins’ (LAMP). In this method, a
gold substrate is functionalized with a binary mixture of OH- and COOH-terminated
thiol to provide a reactive SAM on gold. The COOH end groups enable subsequent
derivatization of the monolayer, while the OH end groups resist non-specific protein
adsorption. The COOH end groups of the mixed SAM are converted to reactive esters,
and covalently bound to a bifunctional amine-terminate oligo(ethylene) linker. The
terminal amine groups in the oligo(ethylene-glycol)-derivatized SAM are covalently
bound to a caged biotin ester. Finally, caged biotin is deprotected by UV light with a
photolithographic mask and the resulting pattern used for the immobilization of proteins
through the same biotin-avidin-biotin bridging method described previously. The main
advantage of this technique is its robustness whereas the high number of processing
steps is clearly its main disadvantage.

Another approach is to combine the use of biotin with the aryl-azide photochemistry.
Pritchard and co-workers have introduced the use of photoactive biotin, i.e., a biotin
molecule with a nitroarylazide group attached to the biotin tail (65). Here, the
photobiotin is immobilized onto the surface via an adsorbed avidin molecule, and the
derivitized substrate is then incubated with a protein solution (IgG). Upon irradiation
through a mask of the solution-covered surface, the free azide group (attached to the
biotin) is transformed into an active nitrene group and inserted into the C-H group of the
protein present in the reaction medium. Repeating this process with four additional
protein solutions allowed assembling five antibodies in five localized regions.

2.4.3.2 Patterning through the antigen-antibody high affinity pair

The antigen-antibody high affinity pair is another protein-ligand system widely used for
patterning through biomolecular recognition. One patterning method that uses the
antigen-antibody pair system has already been discussed in the previous section, i.e.,
patterning antigens over biotinylated antibodies bound to a streptavidin-avidin pattern.
This is an example of a double biorecognition system: the biotinylated antibody has one
ligand that specifically binds to one biomolecule, i.e. streptavidin, and a second ligand that binds to the second biomolecule, i.e. the antigen.

All micro-immunonoassays fall in this category, since they involve the attachment of an antibody over a micro-pattern to selectively immobilize the target, i.e., the antigen. One notorious example is the ‘enzyme-linked immunosorbent assay’ (ELISA) used for the detection of an antigen (or antibody) in a sample. The assay utilises two antibodies for the detection of a target antigen. One antibody is immobilized to a surface, whereas the second antibody carries the label, i.e., an enzyme, which is attached to the antibody via covalent binding or via biotin-avidin linkage. Currently, ELISA is performed in plastic microliter plates, but micropatterning methods will allow in the future the miniaturization of this type of assays (25). Surface-immobilized antibodies can also be used for the immobilization of enzymes (66) and some types of cells (e.g., bacteria cells (67)).

In all those applications, the antibody (or antigen) can be attached to the surface using one of the methods reviewed in this Chapter, i.e., photopatterning, SAMs, direct patterning, etc., and immobilization can be accomplished via spontaneous adsorption or covalent binding. As previously mentioned, the orientation of the immobilised antibody is an important condition. Orientation can be controlled, for instance, by using protein A or oriented monolayers of avidin (or streptavidin).
2.4.4 A new direction: patterning with microspheres

So far, methods that have been reviewed for patterning biomolecules use flat surfaces. Moreover, for most of these methods, the flatness of the surface is an essential prerequisite for the technique to work, as in the case of the SAMs, and photolithographic techniques. Non-planar surfaces, such as microbeads, have been recently introduced as an interesting alternative to the conventional, flat substrate. In this approach a single micropattern with a defined chemical or biochemical functionality is replaced with the surface of a single microbead or a cluster of microbeads.

Commercially, there are microbeads fabricated out of various materials (e.g., silica, polymers like polystyrene etc.), fluorescent in different colours, functionalized with a specific chemical group, e.g., amino, carboxyl, epoxy etc., or with a specific bioligand (e.g., biotin) or protein (e.g., avidin, IgG). Also, beads with specific functionalities or proteins can be custom-built by some manufacturers, and derivatization on the surface bead, such as attachment of a cross-linker (e.g., EDC or NHS) to mediate protein immobilization, are easy to perform. In terms of dimensions, a large selection of sizes is available, which range from tens of microns to tens on nanometres. Thanks to this large selection of microbeads, it is not a problem to find the type with the required surface properties. For protein micropatterning, the challenge is their positional immobilization on a surface.

2.4.4.1 Microarrays of cluster of microbeads

Non-planar substrates have the obvious advantage of having a higher exposed area compared to a planar surface. If an ensemble of microspheres replaces the planar surface, the area for protein attachment is increased, the extent to which depends on the type and number of beads employed. Along this line of thinking, some researchers have considered patterning proteins in an array of microwells filled with microspheres (68). There are two approaches to obtain this type of array. In the first one, photolithographic exposure and etching of a PEG-coated resist surface is used to generate a pattern of microwells. Polystyrene microspheres functionalized with carboxyl groups are then

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3 In this Thesis, microbeads and microspheres are treated as synonymous. When microbeads are not spherical, they are referred to as microparticles.
assembled within the wells and used to covalently bind proteins (Figure 2.5). In the second approach (69), the bead solution is mechanically spotted on a flat substrate in an array format, where is left to dry. Spots of agglomerated latex beads are formed and a PDMS pre-polymer solution poured over the bead-array. After curing, a PDMS-latex bead microarray is formed. In both cases, the result is a bead-microarray where each spot is formed of a variable, immeasurable number of beads, distributed unevenly throughout the spot. Therefore, in both approaches, the array is formed of spots of agglomerated beads where the ‘exposed area’ of the microbeads in each spot is different, i.e., the ligand density of each well is variable and not controllable (Figure 2.5 (middle)). The result is apparent in Figure 2.5 (right), where there is a large variability in the fluorescent emission signal of the immobilized proteins. Thus, the inter-spot variability of microarrays generated with this method represents the main limitation of this technique.

Figure 2.5 SEM (A and B) and epi-fluorescent microscope (C) images of clusters of microbeads arranged in an array format. Reprinted in part from (68) with permission from the American Chemical Society (Copyright 2005).

2.4.4.2 Microarrays of single microbeads

The problem of not being able to know the exact density of the microbeads inside the wells of the array could be solved, in principle, by using an array of single microbeads, i.e., by fabricating microwells where only one bead can be trapped in. This has been achieved by etching an array of microwells on a silicon oxide or on a photoresist
substrate and then by letting the microbeads self-assemble inside the hole either by spin-coating the bead solution (70), (71) or by using a flow cell system (72). The latter method allows complete filling of the microwells, with same number of beads per well (see below), while spin coating is a random deposition method, which leads to incomplete and/or uneven filling of the microwells (Figure 2.6). Patterned polyelectrolyte multilayers have also been used to fabricate 2D colloidal arrays (73), (74).

Figure 2.6 Spin coating (A) and flow-cell deposition (B) of latex microbeads inside an array of microwells. A: reprinted with permission from (70) Copyright 2004 Wiley-VCH Verlag GmbH; B: reprinted with permission from (72) Copyright 2001 Wiley-VCH Verlag GmbH.

Controlling the relative dimension of the micro-hole (or micro-pattern) and the radius of the beads allows fabricating arrays of single beads (Figure 2.7).
A potential limitation of this approach is the mechanical stability of the array, which needs to withstand vigorous washing. Ideally, thus, the beads should be physically entrapped inside the substrate, for instance by using a thermo-responsive polymer. Also, it is logical to expect that an array of single beads would be really advantageous compared to a planar array if the beads were not embedded inside the substrate but standing out from it, i.e., on a post. This would have two advantages: it would allow using most of the microbead surface for immobilization and it would reduce the noise from the background. This platform has not been developed yet.

2.4.4.2.1 The BeadArray™ technology

An important example of a microarray of single beads is represented by the BeadArray™ technology, commercialised by Illumina, Inc., and developed from the work of Walt and co-workers from Tufts University (75), (76). The BeadArray™ technology uses a fibre optical array made from an imaging fibre filled with micron-sized beads. The imaging fibre has a 1.4 mm diameter containing ~ 50,000 individual, ~3.6 μm diameter optical fibres. A wet chemical etching procedure is used to selectively
etch the cores of the individual optical fibres by taking advantage of the difference between the core and cladding materials. The shape and volume of the resulting microwells are controlled by the etching procedure. The microwells are then filled by immersing vertically the etched fibre in a solution of microbeads. Microbeads complementary in diameter to the micro-well size are used. According to the authors, once the microspheres are dispersed in the microwells, they remain in place.

The BeadArray™ technology is an example of a non-positional microarray, i.e., the identity of each element of the array is not defined by its position within it. In this method, the identity of each element is defined by a code inserted inside the beads. The encoding scheme uses fluorescent reporter dyes that are spectrally resolved. For example, a two-dye system can be used to encode microspheres using different concentration ratios of the two dyes, producing sets of microspheres with fluorescent intensities in a distinct “signature” range. The number of resolvable signatures for a given dye pair is determined by the broadness of the ratio range.

Since microspheres are available with various polymer composition and surface functionalities, a large microsphere sensor library could be created with this method. The concept was demonstrated (77) using three different types of microbeads encoded with three “dyes signatures”. The encoded fibre optical sensor was exposed (sequentially) to three solutions of complementary bimolecules (e.g., avidin for the biotin-functionalized beads) labelled with a reporter dye. The reporter dye needs to have emission spectra separate from the ones of the “dyes signature”. After recognition (imaged via the reporter dye), the identity of each microsphere is determined by measuring the emission ratio of the two dyes used for encoding the microspheres.

The BeadArray™ technology has been applied for the detection of a variety of analytes ranging from DNA to organic vapours (78), and is currently under investigation for its application to proteomics. One of the major advantages of this technology is that the packing density of the array is extremely high. However, the necessary decoding step is a major drawback since is limited in the number of unique dye combinations that can be used and successfully differentiated. This limitation could be overcome using semiconductor quantum dots (QDs), nano-sized particles characterized by having narrow fluorescence emission bandwidths and which represent a new class of
fluorophore labels (79). Alternatively, a different encoding system could be used, for instance a geometric code rather a fluorescence-based code.

2.5 **DIRECT PROTEIN PATTERNING**

Most of the patterning methods so far described are based on the selective attachment of proteins over a pre-pattern with controlled chemical properties. Some methods allow to directly pattern proteins in specific locations without the need of fabricating a pre-pattern. Those methods can be defined as “direct patterning” methods, since the proteins are directly printed to a surface from a solution. Patterning occurs either by using a physical stamp (µCP, MIMIC and MEMPAT), the beam of a laser (laser printing) or the tip of a scanning probe (DPN).

2.5.1 **Microcontact printing (µCP)**

Microcontact printing (µCP) was originally developed for printing alkanethiols on gold with spatial control to mediate protein selective immobilization, as previously reviewed (Section 2.4.1.2). The method was later extended by the IBM Research Division in Zurich for printing patterns of proteins and is now a widely used method to generate patterns of proteins, enzymes, adhesion peptides and even bacteria and cells.

The central element of µCP is the stamp. The chemical and physical characteristics have been already discussed at the beginning of this Chapter (Section 2.3.2). Most commonly, a PDMS stamp is used. The stamp is coated with the “ink” solution, throughout rinsed with buffer and water, air (or nitrogen) dried and then placed into conformal contact with a flat, receiving substrate. Typically, the stamp is brought into close contact to the surface at an angle and set down gradually to ensure that conformal contact propagates uniformly. Delamarche and co-workers were among the first to demonstrate that this method allows printing a monolayer of proteins (80). When alignment is needed, a modified mask aligner or home-built printer using step motors is used. Generally, the PDMS stamp used for µCP is untreated, i.e., hydrophobic. When inking cationic polypeptides such as polylysine, it is first necessary to render the stamp hydrophilic.
2.5.1.1 Substrates

The first requirement for the µCP of biomolecules is having a conformal contact between the stamp and the substrate. Therefore, this should present minimum roughness. Substrates that have been successfully used include polystyrene, glass and silanized glass, amphiphilic comb polymers (81), Si wafers, PMMA, and substrates covered with a thin evaporated metal and/or SAM (80), (82), (83)). The printing time using a PDMS stamp does not seem to play a role: printing takes the few seconds necessary to propagate the initial contact to the entire substrate (83).

2.5.1.2 µCP proteins

As anticipated, µCP can be used to directly print proteins to form a micropattern. Many different types of proteins can be inked from an aqueous solution onto a PDMS stamp. The size of the protein does not seem to play an important role in this technology. A wide range of proteins have been patterned with this method, ranging from cytochrome c (12.5 kDa) to fibronectin (440 kDa) (80).

An important difference between µCP proteins on surfaces and µCP alkanethiol on gold is the limited amount of protein physisorbed on the stamp. Whereas alkanethiol can diffuse inside a PDMS stamp in sufficient amounts allowing multiple prints, re-inking the stamp with proteins is necessary after each print.

The details of how and why proteins transfer from a stamp to a surface are still under investigation, but a recent paper highlighted how the difference in wettability by water between the stamp and the surface determines whether transfer occurs (84). It was found that proteins tend to transfer when the substrate is more wettable than the stamp. For instance, transfer from an untreated PDMS stamp was effective on hydroxy, carboxyl and EGₙ-terminated SAMs, whereas surfaces treated with methyl-terminated SAMs resisted µCP (84). These findings are surprising and suggest a difference in the mechanism of protein immobilization by physisorption versus µCP. EGₙ-terminated SAMs resist adsorption but allow printing; in contrast, methyl-terminated SAMs allow adsorption but resist printing. Interestingly, fluorination of the stamp to render it less wettable is an effective method to increase protein transfer on a hydrophilic surface such as glass (84). Therefore, surface modifications that increase the wettability of the
substrate or that decrease the wettability of the stamp expand the types of substrates that can be used for the µCP of proteins, allowing also immobilizing proteins onto surfaces that do not readily adsorb protein from solution, such as glass and PEG.

2.5.1.2.1 Activity of the printed proteins

It was previously discussed (Section 2.2.1) discussed how the adsorption of a protein on a surface, albeit simple to perform, is a complicated phenomenon in which the biological activity of the immobilized proteins might be lost or significantly altered. As noted by Delamarche (83), microcontact printing proteins harbours this risk twice: when the proteins are inked, i.e., adsorbed on the stamp, and when they are transferred to a receiving substrate. While, in principle, the deposition of proteins from solution onto a PDMS stamp should be analogues to that of proteins on hydrophobic surfaces, transferring proteins by printing is more difficult to evaluate. For the transfer to occur, the adhesion of the protein with the substrates must overcome that of the protein with the stamp. As discussed in the previous section, this process is not straightforward and appears to be quite different compared with protein immobilization by physisorption. The printing process could also create a mechanical stress on the protein, which could lead to irreversible conformational changes.

Numerous different proteins have been printed and their activity after printing compared to adsorption from solution (80). In general, enzymes are more susceptible to denaturation during printing whereas immunoglobulines are more robust against loss of function, which explains why µCP is widely used for patterning arrays of antibodies.

2.5.1.2.2 Printing multiple types of proteins

A convenient advantage that µCP has over photolithographic techniques is the ability of printing different types of proteins in a quick and straightforward way. This is achieved with two methods, namely additive and subtractive printing (82). The first allows fabrication of an array of \( n \) proteins using additive patterning steps: once a substrate is microcontact-printed, more proteins can be printed next or over previously printed ones. The latter strategy allows creation of 3D protein-based architectures. No loss of contrast or resolution occurs between printings, i.e., the stamp does not pick up proteins already
printed. At least for proteins, it appears that \( \mu \text{CP} \) is a unidirectional process. The subtractive approach involves first inking a flat stamp homogeneously with the first protein, and then bringing it into contact with a patterned surface (e.g., micromashined silicon). In the regions of contact, the proteins are transferred to the patterned surface (lift-off), opening up areas on the flat stamp for subsequent protein adsorption. The process can be repeated using substrates made of different materials such as silicon, glass or plastic to lift-off patterns of proteins from the stamp (82).

2.5.1.3 \( \mu \text{CP} \) bioligands

\( \mu \text{CP} \) can also be used for printing a ligand to be used for selectively immobilize a protein via covalent attachment or biorecognition. Yang and co-workers have developed a method called MAPS-‘microstamping on an activated polymer surface’ (85). In this multi-step approach, the surface of a polymer is first modified, in one or more steps, to introduce a reactive group of interest. In a subsequent step, a PDMS stamp is inked with a biological ligand containing a complementary reactive group and is brought into contact with the activated surface of the polymer. This results in spatially resolved transfer and coupling of the biological ligand with the activated surface of the polymer. An example of this approach involves the immobilization of a ligand having a biotin-terminus, used to mediate the attachment of streptavidin (86), (81).

2.5.2 Nanocontact printing (nCP)

Soft-lithographic techniques, such as \( \mu \text{CP} \), are currently most useful for patterning features with lateral dimension of 500nm or larger. One of the major challenges for \( \mu \text{CP} \) has been achieving the capability of printing proteins with high resolution, i.e. with later dimension lower than 100 nm.

The main problem associated with stamping proteins with high resolution is the stability of the nano-features of the stamp itself, which, being soft and highly compressible, can deform, buckle and collapse. One way to improve the stability of the patterns is to affix to it a stiff backplane or to change the chemical formulation of the stamp itself, in order to obtain a harder polymer. Both the research groups at IBM-Zurich (87) and at Harvard University (88) have explored alternative siloxane polymers having improved mechanical properties, referred to as \( h \)-PDMS (i.e., hard-PDMS). One strategy is the
fabrication of a composite, two layer stamp that uses a thick (∼3mm) slab of PDMS to support a thin (30-40 µm), stiff layer of h-PDMS. This system allows fabrication of features below 50 nm without collapsing as reported by Li and co-workers (89), who printed 40 nm protein lines. A recent work by Renault and co-workers has demonstrated that using an h-PDMS stamp it is possible to reach the resolution limit of a single protein printed (90). This printing ‘method’ that uses harder stamps (which is based on a new material not on a new nanofabrication method) is referred to as nanocontact printing (nCP).

2.5.3 Other contact- printing methods

There are other two methods that allow printing proteins: contact processing (CP) and affinity contact printing (αCP).

2.5.3.1 Contact processing

Contact processing mimics the deposition of proteins from an aqueous environment to a surface by employing a hydrogel swollen with a protein solution. The capability of CP to generate micropatterns has been demonstrated using a ‘capillary hydrogel stamp’ and a ‘patterned hydrogel stamp’. In the first method, the stamp consists of two parts, a reservoir above the hydrogel containing the protein solution, and a swollen hydrogel embedded in a fine capillary which makes contact with the substrate and mediates the transport of proteins to the substrate (91). Patterning is done in a serial manner. The second method uses a patterned agarose or hydrogel stamp (92), generated by replication of a hydrophobic PDMS master. The stamp is soaked in the desired solution, air dried and printed as in µCP.

The main advantage of contact processing is that biomolecules remain in a biological buffer until the stamp is removed and the substrate dried. This should minimize the chance of protein denaturation. The use of a capillary hydrogel stamp, though, leads to a slow, serial patterning process, whereas a patterned hydrogel stamp has the intrinsic limitation of not allowing high-resolution patterning due to the mechanical instability of the stamp. This method is most useful for cell patterning, where patterns of 1 µm or greater are required (44). For instance, an agarose microstamp was recently used for the direct patterning of bacterial cells (93).
2.5.3.2  Affinity-contact printing

Affinity contact printing (αCP) uses a PDMS stamp with covalently immobilized ligands, which selectively bind receptor molecules from an ink solution (94). The stamp can be roughly compared to a chromatography column due to its ability to extract proteins selectively from a solution. The captured molecules are then released on a substrate during the subsequent printing step.

The main advantage of αCP is its theoretical ability to pattern multiple types of proteins in a straightforward way. Of course, the main limitation is the need to prepare a PDMS stamp with different affinity-sites through numerous cross-linking steps, a task that can become quite operationally challenging. Also, the quality of the patterns may degrade when preparation involves a large number of steps.

2.5.4  Stencil and microfluidic patterning

Some soft-lithographic methods use a physical barrier to limit the area where proteins attach, which is removed after protein immobilization. The methods include membrane-based patterning (MEMPAT) and microfluidic patterning.

The first method uses a perforated membrane made of PDMS that has circular or square holes with different diameters, sides and height of, at least, 50 µm in contact with a substrate. Proteins are patterned within the physical constraints provided by the holes of the membranes (‘micro-wells’) (95), (96).

In the second method, microliter quantities of protein solution are injected into the microchannels of a microfluidic network (μFN) made of PDMS in contact with a surface. The proteins adsorb only on the areas that are exposed to the microflow. The main advantage of this method is that, when using a complex system of independent flowing zones, each accessible through individual reservoirs, different types of proteins can be readily immobilized on a given surface. Delamarche and co-workers were among the first to demonstrate that a μFN could be used to pattern different IgG with good spatial resolution (97). The pattern was subsequently exposed to a cocktail of fluorescently tagged anti-IgG and only immobilization of these based on specific recognition was obtained. Later, the same group reported printing 16 different types of proteins by means of a microfluidic network (82). Recently, the technique was used to
micromosaic immunoassay (µMIA), where a series of ligands and analytes are placed along micrometer-wide intersecting lines, thus providing a mosaic of signals from cross-reacted zones (25).

2.5.5 Laser Printing

Another method that allows direct patterning is laser printing. One of the most versatile methods that employs a laser guidance apparatus to stamp biomolecules and cells is “Matrix-Assisted Pulsed Laser Evaporation Direct Writing”, or MAPLE DW (98). This technique, originally developed at the Naval Research Laboratory (U.S.A.), has been successfully used to simultaneously deposit scaffolding materials, biomolecules and living mammalian cells at the 10- to 100 µm scale, with retention of bioactivity (98).

2.5.6 Dip-pen nanolithography (DPN)

The DPN technique has recently been applied to the deposition of proteins (99). Significant effort has been directed towards the characterization of the resulting protein nanostructures with regard to their activity. Although DPN is an extremely gentle lithographic technique, surface interactions and covalent or non-covalent attachment chemistry could potentially denature some classes of proteins. Researchers in the Mirkin group at Northwestern University who pioneered the method have begun studying the complex issues involved in preserving the biological activity of immunoproteins such as IgG during a DPN experiment (100). Therefore, the use of DPN for protein spatial immobilization is still under investigation.

2.5.7 Direct patterning: discussion

Direct patterning methods have attracted increasing attention in the last few years in particular since their demonstrated ability to reach nanoscale patterning. µCP and, more recently, nCP offer a simple and convenient method to stamp a variety of ‘inks’, from proteins, to peptides and other macromolecules. The method is parallel but, as discussed, can be used also to print different types of inks, even if this might require laborious alignment processes.

The main limitation of the MEMPAT technology is the fragility of the membrane, which, being extremely thin, can be easily deformed or broken when peeling it off the
silicon master. Also, the method is not suitable for fabricating high-resolution protein patterns. Microfluidic patterning (e.g., µMIA) is a versatile patterning technique that allows immobilizing multiple types of proteins on a variety of substrates. The main limitation of this technique is the fabrication of an array where, obligatory, the same protein (or other analyte) is placed along one line.

Laser printing is an interesting method especially for the development of scaffolds for tissue engineering applications. In the context of protein patterning, though, it is a slow, serial, expensive process, therefore it does not respond to the needs of this technology. Finally, DPN combines the advantage of being a nano-patterning tool with the capability of patterning multiple types of inks. Clearly, the main disadvantages of DPN are speed and the possibility of losing protein bioactivity during the printing process. Therefore, µCP or, when high-resolution patterns are needed, nCP are the most versatile direct protein patterning methods.

2.6 SUMMARY

Physiochemical immobilization of proteins in localized, addressable patterns can be accomplished using numerous methods. A surface can either be engineered to have a specific chemical functionality to induce protein immobilization via spontaneous adsorption or chemical bonding, or deposition can be done using a direct approach, like µCP. The two methods can also be used in a complementary way, e.g., microcontact printing proteins on an activated substrate for covalent binding the protein.

Methods that were originally developed for microfabricating electronic circuits (e.g., photolithography) have been adapted for fabricating protein micropatterns, either by applying the technology to engineered surfaces (photopatterning) or by using microlithographic-like materials (photoresists). Among the most popular surfaces for protein patterning are SAMs of alkenethiols on gold, which can be used for generating micro-patterns with distinct functionalities. A drawback of SAMs of alkenethiols on gold is their instability to temperature and the UV component of light.

Among the direct patterning methods, µCP has received most attention due to its simplicity and versatility of patterning various proteins while maintaining their
biological activity. Nanoscale resolution was recently demonstrated using an alternative formulation to PDMS, the material normally used for fabricating the stamp used for µCP. Other soft-lithographic techniques, like microfluidic patterning, are also often employed for patterning proteins.

Together with surface engineering, the natural complementary of some biomolecules can be exploited for the patterning of proteins. The interaction of biotin-avidin and antibody-antigen can be used not only for demonstrating the bioactivity retention of a protein pattern, but also as a mean to control protein selective immobilization. Methods that use biorecognition as a patterning tool often combine the ligand affinity mechanism with a surface engineering method, such as the use of caged photobiotin.

Finally, recently the use of non-planar substrates in the form of microbeads has been introduced. Microbeads can be organized in an array either as spots of agglomerated beads or as single beads. Illumina, Inc. has commercialised a sensor system that uses a bundle of optical fibres filled with microbeads (BeadArray®). The sensing elements (i.e., the beads) are not spatially addressable so a code is inserted in each bead in the form of a “dye signature”. Decoding of the sensor after recognition of the target/s is necessary and represents the main drawback of this technology due to the limited number of dye combinations that can be successfully used. The problem could be solved using different encoding dyes (e.g., QDs) or a different encoding method (e.g., non-fluorescent based).
2.7 PROTEIN TOPOGRAPHICAL PATTERNING

Topographical patterning is an alternative to physiochemical and biochemical patterning in the sense that, whereas in those methods protein immobilization is spatially controlled through electrostatic, chemical or biochemical interactions on a planar substrate, in topographical patterning a profiled feature allows patterning by exploiting the depth of a ‘groove’. Methods used to generate profiled patterns (e.g., laser ablation) often induce alteration of the surface properties, therefore topographical and physiochemical patterns often coexist. For this reason, as will be discussed throughout this section, it is important to consider the chemical nature of a topographical pattern.

In the context of protein micropatterning technology, profiled features have the advantage over planar patterns of minimizing the inter-spot contamination and the drawback of more difficult access to the recognition biomolecule in a microconfined area (e.g. antigen for antibody microarrays). An optimal solution is the use of a shallow profile feature, which takes advantage of the benefit of the former and mitigates the latter.

Surfaces with ordered or randomly dispersed nano-topographies are also of interest to investigate fundamental aspects of protein adsorption to surfaces. As it will be discussed, though, this matter is still under extensive investigation and, at the time of writing, no conclusive information is available on the effect of surface nano-topography on protein adhesion and bioactivity retention.

2.7.1 Protein patterning in profiled shallow features

One method to generate profiled but shallow micro-features is the ablation of a sensitive substrate. This is a direct writing approach that has the main advantage of being serial, therefore allowing patterning numerous types of proteins. Few works have used this method to generate patterns of proteins. Generally, a bilayer system is used, i.e., a protein adhesive layer covered with a protein resistant layer which is selectively removed via laser ablation. For instance, Thissen et al. reported the fabrication of adhesive and non-adhesive patterns through laser ablation of a PEO surface (101). A protein adhesive layer made of an allylamine plasma polymer (ALAPP) was first coated with a PEO layer and then selectively removed through direct laser ablation using an
excimer laser, leaving the desired pattern. The pattern was then used for mediating cell attachment. Cells, mediated by serum proteins, attached selectively on the adhesive pattern, with a complete lack of attachment on the non-adhesive pattern.

Brizzolara (102) has reported and patented an alternative method to pattern multiple types of proteins, specifically antibodies, using an ablative method. Here, the adhesion-promoter layer is a polystyrene surface, which is coated with BSA. Argon ion beam lithography was then used then to remove the BSA coating, leaving a clean, polystyrene pattern (as confirmed by XPS analysis) where the proteins were adsorbed. Brizzolara reported adsorbing different antibodies on the polystyrene pattern in a serial manner and demonstrated their preserved bioactivity by selectively binding fluorescently labelled antigens from a mixed solution.

Similarly, another work (103) reported the use of a bilayer system made of polyimide (PI) and a protecting layer of either BSA or a low-temperature polymer laminate. Here, the protective layer was removed via laser ablation using a 193 nm excimer laser and the laser-defined regions used to adsorb avidin. Labelled biotin was subsequently adsorbed on the pattern for visualization and to demonstrate the bioactivity retention of the adsorbed avidin.

Finally, Nicolau and Cross (104) have reported patterning proteins in profiled features generated through the selective irradiation (not ablation) of a UV photoresist with the use of the laser of a conventional confocal microscope. Here the bilayer system used is made of a layer of an e-beam photoresist (PtBMA) covered by a layer of UV photoresists (DNQ/Novolak). The main difference of this work from the previous ones is that instead of using a bilayer system made of a protein-blocking/protein adhesive material and selective ablation of the first one, here the profiled features and selective adsorption is achieved using bilayer photolithography. Specifically, the DNQ layer was irradiated with the 488 nm laser of the microscope and developed. After flood exposure of the pattern the unexposed DNQ/Novolak regions were rendered highly hydrophilic, leaving hydrophobic PtBMA spaces where labelled avidin was selectively adsorbed and fluorescently visualized. The final result was a positive tone protein pattern and the system referred to as ‘protein on bottom’ mode. This photoresist bilayer was also used in this same work to fabricate a negative tone, ‘protein on top’, pattern. In this case, the
surface treatment aimed at making the photoresist DNQ/Novolak lines as hydrophobic as possible and making the bottom channels as hydrophilic as possible. The two systems were compared and it was found that the first, ‘protein on bottom’ mode was the one capable of giving higher resolution (2 \( \mu \text{m} \) vs. 5 \( \mu \text{m} \)), higher contrast and best control over the process.

Some of the above mentioned works use a photoablation process, which is known to be able not only to create structures but also to change basic chemical properties of the resulting surface. Properties such as hydrophobicity/hydrophilicity, charge and the formation of new functional groups can be altered under various ablative conditions (35). Numerous studies have investigated the chemical characteristics of surfaces before and after ablation with XPS and found that, in general, the ablated surfaces are less charged and more hydrophobic. Also, enhanced surface roughness is characteristic for polymers after photoablation (35) (Figure 2.8). This opens an interesting question, i.e., if the increased surface roughness of, for instance, an ablated polymer micropattern influences the adsorption of proteins.

Figure 2.8 Various undesirable effects associated with laser ablation. Reprinted with permission from (34), Copyright 2002 CRC Press LLC.
2.7.1.1 Proteins sensitivity to surface nano-topography: an open question

The surface chemistry of a substrate has a strong influence on the absorption process (21). Proteins mediated the adsorption of cells to surfaces (15), and there is evidence to say that nanoscale topography influences cell adhesion and behaviour. Therefore, the surface nano-roughness should play an important role in the amount and bioactivity of adsorbed proteins. Surfaces with nanometre-scale roughness (i.e. with nanotopographies in the same order of magnitude of protein dimensions) should be of particular significance. Therefore it would be reasonable to expect that surfaces with nano-roughness should influence, e.g., increase, protein adsorption compared to smooth surfaces. Nevertheless, published information regarding protein adsorption on textured surfaces with surface nano-topographies is contradicting.

As previously discussed, Schwartz et al. (103) have reported protein patterning on laser ablated contoured features. In this work, polyimide (PI) was used as the light absorbing polymer. Protein (BSA) absorption on ablated and un-ablated PI was quantified using a radiolabel method, i.e. through the analysis of the $^{14}$C-labeled protein. It was found that up to three times more protein was adsorbed on the ablated PI in comparison to the non-ablated surfaces. This difference was attributed to an increased surface hydrophobicity and roughness on the ablated surface, even though a nano-topographical analysis (e.g., AFM) was not reported. Also, as the authors highlighted, this result is only an indication of the total amount of protein adsorbed, but it does not give information regarding the protein bioactivity. The authors demonstrated protein bioactivity retention on ablated PI regions using the avidin-biotin recognition method, but did not use an untreated control surface to assess if the surface topography of the ablated PI had an effect on the avidin adsorption.

Recently, a work was published on protein adsorption on model surfaces with controlled nanotopography and chemistry (105). Here, the absorption of collagen was quantified on a smooth, control surface and on a rough surface generated by colloidal lithography. The effect of surface chemistry was assessed by functionalization of the smooth and rough surface with methyl- and hydroxy-terminated SAMs, which gave, respectively, a hydrophobic and hydrophilic surface. Hydrophobic surfaces (i.e. methyl terminated) were found to absorb larger amount of collagen compared to hydrophilic surfaces. Interestingly, on rough substrata the adsorbed amount of protein was found similar to
the absorption on smooth substrata. Surface topography was found to affect the film morphology, with elongated supermolecular assemblages formed on smooth surfaces, structures that were not observed on rough substrata. Therefore, in this work, while the absorbed amount of collagen was not affected by the surface chemistry, the supermolecular organization of the absorbed layer was found to be controlled by both surface chemistry and topography.

The need to investigate the sole effect of surface topography on protein adhesion has taken Han and co-workers (106) to use commercially ultrafiltration membranes made of poly(ether sulfone) (PES). In this work the absorption of hen egg lysozyme was investigated on various membranes (compositionally identical) with different surface roughness characteristics. Interestingly, at the nanolength scale considered in this work (surface roughness between 5 and 60 nm) the roughness had little effect on the amount of adsorbed protein when considering the actual surface area of the substrate available for absorption. Moreover, no significant changes in the protein secondary structure were observed with increasing roughness.

No definitive conclusion on the effect of surface topography on protein adhesion can be inferred from these works for a number of reasons. Firstly, each work investigates a different type of protein (BSA, collagen and lysozyme), which are dramatically different in secondary structure and in molecular weight. Secondly, each work looks at surfaces with different chemistries, (PI, SAMs and PES), which will definitively have an important effect on the protein adsorption behaviour. Finally, the methods used to generate surface roughness are significantly different (e.g., laser ablation and colloidal chemistry), therefore the nature of the surface roughness is different in each case.

The effect of surface topography on protein adhesion remains, thus, an open question. As observed by Han et al. it is possible that only when the average length scale of the substrate surface is of the same size or smaller than the dimension of the protein under investigation the surface roughness is likely to influence the absorption kinetics and the amount absorbed (106).
2.7.2 Summary

Surfaces that are engineered to have micro- or nano-features for immobilizing proteins are fabricated with methods that, often, induce a change in surface chemistry. Also, nano-surface roughness is often produced, which can influence protein adsorption. Thus, when using a topographical method for generating patterns where selectively immobilize proteins, the question of if and how the nano-surface roughness influences the adsorption process arises. The literature information regarding protein sensitivity to surface nanotopography is not univocal and suggests that the topic requires further research.

2.8 Conclusions

The immobilization of proteins on surfaces can be achieved using different immobilization strategies, namely nonspecific adsorption, covalent binding and high-affinity ligand pairing. Each of these methods introduces specific advantages and challenges; therefore there is not a unique method for protein immobilization, nor a general, ‘magic’ substrate that can effectively immobilize any protein with retention of its bioactivity. The complexity of the interaction between proteins and surfaces requires that each immobilization methodology and type of substrate used, to be investigated for the specific application it is designed for.

The vast diversity of proteins and the complexity of protein immobilization to surfaces have triggered an extensive research for developing suitable micropatterning methods. There are numerous strategies that can be employed for the fabrication of a surface micropattern, all sharing the use of a microfabrication method. Some methods have been adapted form their original field of application, while others has been developed specifically for integrating biomolecules and inorganic materials at the micro-level.

Microlithography was the first technology to be adapted to fabricate patterns of proteins with micro-scale resolution. Tow methods were developed, silane-coupling micropatterning and photopatterning, both variations of photolithography. While silane-coupling patterning uses organic solvents in the fabrication process (to remove the photoresist), photopatterning avoids it, since it exploits the change in surface chemistry arising from the irradiation with light, most of the times through a mask. Materials that
are used in photopatterning include photoresists, photopolymers and photosensitive SAMs, all sharing the need to be deposited or assembled over a flat substrate, an imperative requirement in contact-mode photolithography. Light (either UV or deep-UV) is used to selectively modify the chemical properties of a surface and specialized chemistries (e.g., arylazide chemistry) are used for protein immobilization. Among the surfaces that have been employed, SAMs are particularly advantageous because they inherently offer a high degree of control in the vertical direction at molecular level. In general, though, the use of photolithography (or variations, such as photopatterning) requires the access to specialized equipments, which are mostly foreign to biological laboratories, as well as specialized chemistries. In the last decade research has focused on finding alternatives to this classical approach to protein patterning, in order to avoid the use of costly microlithographic equipments and bio-hazardous conditions, but also to have access fast, ‘easy-to-perform’ methods. Soft-lithography was introduced to fulfil this need, and is now one of the most employed micropatterning methods used in biology. In particular, μCP has proved to be an excellent method for protein patterning, with bioactivity retention: for this reason μCP was chosen as one of the microfabrication methods employed in the work reported in this Thesis.

A different approach to protein micropatterning is the use of microbeads: instead of creating micropatterns with localized functionalities, functional microspheres are used. In this approach, the challenge is the spatial organization of the microbeads on a surface, to form a mechanically-stable pattern suitable for protein immobilization (i.e., as positional microarray of beads). The use of microbeads represents the base of a cutting-edge commercial product, the BeadArray™ (Illumina, Inc.), which is a non-positional array where the identity of each bead is defined by a colour-code inserted in the microbead. In the present Thesis, soft-lithography together with microbead self-assembly was used to create a single bead positional microarray.

Finally, topographic patterning is a method where patterns, in the form of grooves, are formed for immobilizing proteins. Shallow patterns are the most useful, since they reduce inter-spot contamination without interfering with the detection of the protein inside the pattern. Laser microablation is the microfabrication method that is commonly used, and surfaces range from polymers to bilayers, where one layer prevents protein adhesion and the second, underlining layer (which is revealed upon ablation) favours
protein adhesion. The surface properties of the microfeatures generated with this method are at times altered (depending on the type of laser and surface used), with formation of numerous ablative products not easy to characterize; therefore protein immobilization in ablated features is normally done via adsorption. Upon ablation, the surface topography is also altered, which triggers the question of its influence on protein adsorption itself. This issue is still under investigation, and literature information is not easy to compare due to the numerous nanofabrication methods employed and proteins that are tested to clarify this interaction. The advantage of laser ablation is that it is a serial, direct technique, therefore leaving freedom to create patterns of any shape. When using some substrates (e.g., polystyrene), though, expensive, high-energy exposure tools are needed, which can be difficult to access and costly to operate. In the work reported in this Thesis, laser microablation was employed as a microfabrication tool but a UV sensitive bilayer substrate was engineered, thus allowing the use of an instrument often employed in cellular biology equipped with a UV laser.
3 EXPERIMENTAL METHODS

This Chapter gives details of the microfabrication and characterization methods that were used in the work reported in this Thesis. At the end of the Chapter, some general considerations regarding the conditions employed for protein immobilization are also discussed.

These methods were used for the fabrication of spatially addressable protein micropatterns, with results reported and discussed in the Experimental Chapters 4, 5 and 6. Additional and specific details of the methodologies employed in each work are given at the beginning of the correspondent Experimental Chapter.

3.1 MICROFABRICATION METHODS

3.1.1 Computer-assisted laser writing

Chapter 2 discussed how photolithography employed as a surface patterning tool for biomolecule immobilization uses either UV sensitive polymers (e.g., DNQ/Novolak), which are exposed using mask aligners and broadband UV sources, or polymers sensitive to higher energies (e.g., PMMA or CAPs). One possible solution would be the use of UV sensitive substrates to be patterned with equipments easily encountered in biological laboratories equipped with UV sources, e.g., mercury lamps or lasers. One example of this approach is the use of a commercial microscope used for projection photolithography (MPP), where the microscope mercury lamp is used as the exposure source (107). Another interesting possibility, already discussed in Chapter 2, is the use of the laser of a laser scanning confocal microscope for patterning UV sensitive polymers (104). This instrument is further discussed later in the Chapter. Most software packages that interface with a confocal microscope allow the use of a region of interests (ROI) to expose a selected area of a specimen. Various ROIs can be drawn and simultaneously selected, each having an arbitrary geometry, and each ROI can be set to use a defined percentage of the laser power. Therefore, a “mask” can be programmed
with areas having arbitrary dimensions and transmission. This allows, for instance, the formation of a surface gradient when using a “mask” made of a series of consecutive ROIs with increasing laser powers. Figure 3.1 is an example of a DNQ/Novolak surface patterned with this method.

![Figure 3.1](image)

**Figure 3.1** Laser scanning confocal image of a DNQ/Novolak surface exposed through a series of 21 ROIs. Scale bar is 50 µm.

The sample in Figure 3.1 was fabricated by spin coating a photoresist (AZ 1500, Clariant) over a cleaned glass slide, which was then placed face down on the inverted microscope stage and exposed with the 488 nm laser line of the confocal microscope, using a series of ROIs (each being 50x30 µm). The laser intensity was varied from 0% (top left of image-no darkening detectable) to 100% (bottom left), with increment of 5% between each ROI. During exposure, the UV component of the resist (i.e., the azide group) is lost which is reflected in decreased fluorescence, due to loss of conjugated double bonds, therefore the higher the energy of the laser, the higher the darkening of the exposed area (as shown in Figure 3.1).

The use of a confocal microscope for exposing photosensitive surfaces is promising and cost-effective, since it avoids the need to fabricate a photomask. This method, though, presents a major limitation: imaging is done using the same confocal microscope, therefore using a laser, which is likely to expose the surface of the photoresist. For
instance, the image in Figure 3.1 was acquired using the 543 nm laser line. The wavelength can be chosen to minimize undesired exposure during imaging but, unless the material used is sensitive to only one wavelength (which is not the case of DNQ/Novolak), damaging is still likely to occur. This is expected to occur with most UV photopolymers, which are sensitive to a UV band rather than a single wavelength. Also, the micropattern is fluorescent, which represents a problem if fluorescent proteins are adsorbed and fluorescence microscopy used as the detection method.

An interesting alternative is the use of a LaserScissors®, a system that transforms an inverted microscope into a powerful laser microdissection instrument. The system, illustrated in Figure 3.2, consists of an inverted microscope adapted with a computer-controlled, pulsed UV laser. The Laser Scissors Workstation® (Cell Robotics, Inc.) allows the observation of the specimen directly on the computer screen, where the position of the laser focal point is clearly indicated. A software controls both the function of the stage (position, speed) and of the laser (laser power, firing rate). The user can program a tab-delimited text file containing the relevant settings (laser on/off, power, firing rate, coordinates, stage speed) and supply it to the software. The laser power can be computer controlled from 1 to 100% of maximum power, and the firing rate varied between 1 to 20 pulses/s, while stage speed can be varied from 0 to 250 μm/s.
The LaserScissors® is focused using the objective lens of the microscope to form a strong laser microscalpel at the laser focal point. The laser focal point is used to irradiate a target of interest as the sample is moved with the microscope stage. As a microdissection tool, the LaserScissors® has found numerous applications in biological sciences at the cellular level (e.g., membrane perforation) and subcellular level (e.g., ablation of specific intracellular organelles) (108), (109).

3.1.1.1 The LaserScissors® as a surface micropatterning tool

Vaidya (110) has described the use the LaserScissors® for fabricating a chromium photomask, which was then used for exposing a photoresist to be employed as a master for PDMS molding. The same authors reported the use of the laser of a LaserScissors® for selectively desorb areas of a SAM assembled over a gold coated glass slide (110). Tuning the energy of the laser ensured only desorption of the SAM and avoided ablation of the underlying gold layer. A second thiolsilane was then adsorbed over the exposed
gold regions and the resulting pattern used for biomolecule spatial immobilization. In these works the LaserScissors\textsuperscript{®} was used either as an ablation tool (fabrication of a photomask) or as a surface-patterning tool (selective SAM desorption), depending on how the intensity of the laser was tuned.

The LaserScissors\textsuperscript{®} is therefore a versatile tool for the fabrication of patterns for biomolecule patterning, but while the fabrication of surface patterns has been demonstrated, fabrication of profiled features using the LaserScissors\textsuperscript{®} system suitable for protein patterning has not been demonstrated yet. In the present Thesis, this capability was investigated and demonstrated using a multi-layer substrate, ablatable with a UV laser but not fluorescent; this work is presented and discussed in Chapter 4.

### 3.1.2 PDMS replica molding

Soft-lithography is the second microfabrication method that was used in the present work. The central element of this technique is the PDMS mold, fabricated via a process called replica molding. In the work presented in this Thesis, the PDMS stamp was used both as a patterning tool (Chapter 5) and as fabrication tool (Chapter 6).

The most commonly used material to fabricate the mold is Sylgard\textsuperscript{TM} 184 supplied by Dow Corning. This commercial material is sold in a two-part kit. The first part contains vinyl terminated polydimethylsiloxane chains of about 60 monomers long \((n=60)\), also called the base agent. The second part contains a ready to use mixture of methylhydrosiloxane dimethylsiloxane copolymers of about 10 monomers long \((m=10)\) and a platinum based catalyst known as Karshtedt catalyst: 
\[
\text{Pt}^{1.5}((\text{CH}_2=\text{CH}(\text{Me})_2\text{Si})_2\text{O}) \quad (111).
\]
This second part is often referred to as the curing agent. The precursors for PDMS synthesis are shown in Figure 3.3 (a). The manufacturer recommends mixing the two part in the ratio 10:1 \((10:1 \, (v/v) \, \text{base: curing agent})\). Once the precursors are mixed, an organometallic catalysed cross-linking reaction is induced, schematically represented in Figure 3.3 (b). During mixing air bubbles are formed, which need to be removed prior curing. The mixture is therefore placed under vacuum (down to 0.1 Torr) for about 1 hour. The precursor components have low viscosity; thus degassing only removes air bubbles.
To fabricate a PDMS mold, the polymer precursor is gently poured over a master, which contains the desired microfeatures to be replicated (of which a reverse image is obtained). It is important to avoid the formation of air bubbles while pouring the precursor over the master but, if this occurs, the sample can be re-degassed. The master is generally fabricated via a photolithographic method and is made of silicon or photoresist (e.g., SU-8, a negative tone photoresist). Irreversible adhesion of the polymer to the master can be a problem, especially when using an SU-8 master, in which case an anti-adhesive surface treatment of the master is required, such as fluorination. This is done by placing the master in a desiccator under vacuum with a vial containing few drops of a tricholorosilane (e.g., 1,1,2,2-terahydrooctyl-1-tricholosilane).
Curing is normally performed at 60° C for one or two hours, but shorter curing times are possible increasing the temperature to 150° C (15 minutes). PDMS can be cured at room temperature but takes at least 24 hours. No harmful phenomenon of evaporation occurs during the curing step, which, therefore, can be performed in a normal convection oven. After curing, the polymer can be easily peeled off from the master. In applications where contact of the stamp with biological species is foreseen, over-curing the PDMS mold is recommended, since residual, uncured materials are toxic (96). In our laboratory we have noticed this harmful effect when working with fungal cells. Over-curing is done by leaving the mold overnight at 65° C after its removal from the master.

Figure 3.4 (a) shows an image of a SU-8 master fabricated via photolithography and the correspondent PDMS replica (Figure 3.4 (b)). The master was fabricated by spin coating SU-8 (SU-8 25, MicroChem, Corp.) over a cleaned 24x24 mm glass slide pre-treated with 3-glycopropyl trimethoxysilane (GPTS, Sigma Aldrich) to ensure good adhesion of the photoresist. SU-8 is very viscous; therefore spinning was performed in two steps: a resist puddle was placed over the glass slide and the slide spun at 500 rpm, ramping at 100 rpm/sec, to ensure a slow, uniform distribution of the resist over the slide. Then, a ramp of 300 rpm/sec to 3000 rpm was set, and spinning at this speed kept for 60 seconds. The resist-coated slide was pre-baked at 65° C for 10 minutes, then ramped to 95° C and baked for other 30 minutes. Pre-baking is necessary to evaporate the solvent and densify the film. A stepping pre-baking method is recommended by the manufacturer to obtained best resist-to-substrate adhesion. Due to the high viscosity of the material, a rim of photoresist forms around the glass slide edges, which therefore was carefully removed before the photolithographic step, to ensure a tight adhesion with the photomask. The resist-coated slide was then exposed through a chromium mask for 60 seconds using a contact aligner equipped with a mercury lamp. Following exposure, a post exposure bake (PEB) is required to selectively cross-link the exposed portions of the film. SU-8 readily cross-linkers and can result in a highly stressed film, leading to cracking. To minimize the stress, the manufacturer recommends performing PEB using a slow ramp or a two-step process. Here, the polymer film was first placed in an oven for 3 minutes at 50 °C, then ramped at 95 °C and baked for 15 minutes. Finally, the slide was developed in PGMEA, under vigorous agitation, for about 2 minutes. SU-8 is a negative photoresist, so the reverse image of the mask design is revealed after the post-bake and development treatment.
The SU-8 master in Figure 3.4 (a) was used as the master for PDMS molding. The master was first fluorinated as described above; then, the PDMS liquid precursors were mixed in the ratio 10:1 (v/v) (base: curing agent), degassed for 1 hour, and gently poured over the master. After curing at 65 °C for two hours, the PDMS was carefully peeled off from the master (Figure 3.4 (b)).
Once a PDMS stamp is formed, it can be used in diverse ways for patterning biological materials. Chapter 5 describes a novel protein micropatterning method that uses a PDMS stamp, termed microcontact printing trapping air (µCPTA). This new stamping method is based on µCP and exploits one of the major limitations of some PDMS stamps, i.e., their instability and tendency to collapse.

3.1.2.1 Material transfer in replica molding

PDMS molding is normally done to replicate with maximum fidelity a master and obtain its negative image. The surface of the master is usually thoroughly cleaned before pouring the polymer precursor, in order to free it from dust or other undesired material that would otherwise be transferred during the replication step. This ‘material transfer’ capability is well known, but it is mainly considered an inconvenient phenomenon, which interferes with the outcome of the PDMS mold quality. Very few works in the literature suggest transferring, on purpose, a material from a surface during PDMS replica molding to form a composite PDMS. One work that uses this approach was previously discussed in Chapter 2 (Section 2.4.4.1), the formation of an array of spots of latex beads embedded in a PDMS mold (69). This involves mechanically spotting a solution of latex beads on a flat substrate in an array format. This platform is then used as the master for PDMS molding, with the beads remain entrapped in the resulting PDMS stamp.

The limitation of this approach was discussed already in Chapter 2, i.e., the array is formed of spots each having a variable number of beads, which cannot be quantified, distributed unevenly throughout the spot. Also, beads are firstly coated (for instance, with an antigen covalently bound to the bead surface), then spotted and finally covered with a PDMS pre-polymer solution, which is cured at 90\(^\circ\). Thus, the coated beads are subject to baking, which is likely to have an adverse effect on the bioactivity of the coating (i.e., immobilized proteins). Indeed, the authors report loss of antibody-binding capacity when a biorecognition experiment using the bead-array was done.

Developing from this work and taking into account its limitations, the present Thesis introduces a novel method for the fabrication of a single-bead, PDMS composite array. This novel platform is termed ‘BeadMicroArray’ and combines knowledge from bead self-assembly in microfabricated structures and PDMS replica molding. The fabrication
and application to protein patterning of this novel microarray platform is presented and discussed in Chapter 6.

3.2 CHARACTERIZATION METHODS

There are numerous methods for the imaging and characterization of protein micropatterns, some well established, other emerging. Advanced microscopy methods (e.g., Atomic Force microscopy (AFM)) and advanced spectroscopy methods (e.g., X-ray Photoelectron Spectroscopy, (XPS)) are used for the imaging and characterization of these types of samples. Each of these methods has its advantages (e.g., resolution) and limitations (e.g., the dimensions of the area that can be scanned), some requiring the use of equipment easy to operate (e.g., confocal microscopy) and others requiring highly specialized machines (e.g., XPS) most of the time operated by dedicated technicians or researchers.

The work reported in this Thesis was entirely done at the Bioengineering Laboratory at the Swinburne Industrial Research Institute, which is equipped with state-of-the art microscopes. The following sections provide a brief description of this equipment, with a focus on identifying their capabilities in the context of imaging surface and protein patterns.

3.2.1 Fluorescence microscopy

The most common method for the detection of protein micropatterns is fluorescence microscopy. Most proteins are not naturally fluorescent (with exceptions, like the large green fluorescent protein (GFP) from the jellyfish *Aequorea victoria*); therefore proteins require labelling with a fluorescent probe prior imaging. Fluorescent tags are small molecules that can be easily covalently bound to a protein (most of the time, its amine-terminus) without altering the protein conformation and, consequently, its bioactivity.

Fluorescence spectroscopy is often used in experiments that investigate the bioactivity retention of patterned proteins, assessed via biomolecular recognition. The protein under investigation is first patterned unlabelled (e.g., an antigen), then exposed to a complementary biomolecule, modified with a fluorescent tag (e.g., labelled antibody). If
biorecognition occurs, a fluorescent pattern is imaged, which is compared to a control sample.

Fluorescent protein micropatterns are often imaged using an epi-fluorescent microscope, where the light source is a high-pressure mercury or xenon lamp (112). An excitation filter allows only the desired wavelength from the lamp to illuminate the specimen. Illumination occurs over the entire sample simultaneously. The light emitted by the sample is collected by the same microscope objective and transmitted through the dichroic mirror. An emission filter finally selects the desired wavelength of the light before it is detected. The fluorescence can be followed in real time using the eyepiece or a CCD camera.

In traditional epi-fluorescence microscopy, objectives focus a wide cone of illumination over a large volume of the specimen, which is uniformly and simultaneously illuminated. A majority of the fluorescence emission directed backwards the microscope is gathered by the objective and projected into the eyepieces or detector (113). The result is a significant amount of the signal due to emitted background light and auto-fluorescence originating from areas above and below the focal plane, which seriously reduces resolution and image contrast.

Images with higher resolution and lower background noise can be acquired using a laser confocal microscope, which is the system that was used in the work reported in this Thesis. The confocal microscope uses a laser (i.e., a monochromatic light) as the excitation source, which eliminates the need of excitation filters. A detailed description of this complex instrument is beyond the scope of this Thesis, therefore only the basic operation principles will be described.

3.2.1.1 Confocal Microscopy

Confocal microscopy relies on point illumination using a highly focused continuous laser beam (113). The laser illumination source is first expanded to fill the objective rear aperture using a pinhole aperture, and then focused by the lens system to a very small spot at the specimen focal plane. The size of the illumination point ranges from about 0.25 to 0.8 μm in diameter (depending on the objective numerical aperture) and 0.5 to 1.5 μm deep at the brightest intensity (113). Figure 3.5 compares the typical
illumination cones of a widefield and point scanning confocal microscope at the same numerical aperture.

**Figure 3.5** Widefield versus confocal microscopy illumination volumes. Reprinted from: [http://www.olympusfluoview.com/theory/confocalintro.html](http://www.olympusfluoview.com/theory/confocalintro.html), with permission from Olympus (Australia).

In confocal microscopy, the emitted light from the sample is collected by the microscope objective and is focused on an adjustable pinhole (Figure 3.6). The focal plane of the objective and the pinhole are confocal planes, meaning that only the light originating from the imaging plane can pass through the pinhole, hence the term *confocal*. The intensity of the emitted light is measured using a photo multiplier tube and translated into an integer number, and a computer records the value and position of the laser beam. An image of the object is finally constructed by either moving the laser beam by pivoting mirrors (Laser Scanning Confocal Microscopy, LSCM) or by moving the entire sample (Stage Scanning Confocal Microscopy, SSCM).
Figure 3.6 Schematic set-up of a laser scanning confocal microscope. $M_x$ and $M_y$ are pivoting mirrors.

By using the confocal pinhole much of the out-of-focus light is eliminated. Objects above and below the image plane do not contribute to the detected signal, resulting in lower background noise, better spatial resolution and increased contrast.

A confocal microscope is equipped with photo multiplier tubes that can accumulate or average the signal over a long period of time, thus compared with epi-fluorescence microscopy, the sensitivity in confocal microscopy is increased. The instrument can be equipped with the possibility of different lasers as excitation sources using a tuneable acousto-optical filter. In combination with two or more detectors, emission filters and dichroic mirrors, this set-up allows to separate the emitted signals from a sample stained with multiple dyes.
The confocal microscope is probably most famous for its ability to image a single plane (0.5 to 1.5 micrometer thin) in a thick sample (up to 50 micrometers or more). By continuously changing the distance between the microscope objective and the sample after each scan it is possible to do optical sectioning of the sample. A software then combines all the optical sections, forming a complete three dimensional image of the sample and its interior.

Additional advantages of scanning confocal microscopy include the ability to adjust the magnification electronically by varying the area scanned by the laser without having to change the objective. This feature is termed zoom factor and can be used to adjust the image spatial resolution. Also, as already mentioned at the beginning of the Chapter, it is possible to scan only a selected region of interest (ROI) or multiple ROIs, and chose for each ROI a specific laser and intensity to be used during the scan.

Another advantage is the digitization of the sequential analog image data collected by the confocal microscope photomultiplier. The continuous voltage stream is converted into discrete digital increments that correspond to variations in light intensity. This way, images can be readily prepared for print output and, in carefully controlled experiments, quantitative measurements of spatial fluorescence intensity can be obtained from the digital data.

The main disadvantage of confocal microscopy is the limited number of excitation wavelengths available with common lasers (referred to as laser lines). Table 3.1 summarises the types of lasers used in confocal microscopy and their relative lines of excitation. The table also gives the emission wavelengths for the lamps used in epi-fluorescence microscopy.
Table 3-1 Laser and arc-discharge spectral lines in widefield and confocal microscopy. Adapted from (113) with permission from Olympus (Australia).

<table>
<thead>
<tr>
<th>Laser type</th>
<th>UV</th>
<th>Violet</th>
<th>Blue</th>
<th>Green</th>
<th>Yellow</th>
<th>Orange</th>
<th>Red</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ar-Ion</td>
<td>351,364</td>
<td>-</td>
<td>457, 477, 488</td>
<td>514</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Blue Diode</td>
<td>-</td>
<td>405, 440</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Diode-Pumped Solid state</td>
<td>355</td>
<td>430, 442</td>
<td>457, 473</td>
<td>532</td>
<td>561</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>He-Cd</td>
<td>322,354</td>
<td>442</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Kr-Ar</td>
<td>-</td>
<td>-</td>
<td>488</td>
<td>-</td>
<td>568</td>
<td>-</td>
<td>647</td>
</tr>
<tr>
<td>Green He-Ne</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>543</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Yellow He-Ne</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>594</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Orange He-Ne</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>612</td>
<td>-</td>
</tr>
<tr>
<td>Red He-Ne</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>633</td>
</tr>
<tr>
<td>Red Diode</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>635, 650</td>
</tr>
<tr>
<td>Mercury Arc</td>
<td>365</td>
<td>405, 436</td>
<td>546</td>
<td>-</td>
<td>579</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Xenon Arc</td>
<td>-</td>
<td>467</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

While a laser emits a monochromatic light, mercury or xenon lamps provide a full range of excitation wavelengths in the UV, visible and NIR regions. To overcome the limited number of laser lines available, fluorescent dyes that carefully match these lines have been developed, such as the Alexa Fluor® series dyes commercialized by Molecular Probes (Invitrogen, Inc.).

The scanning confocal microscope used in this Thesis is the Olympus Fluoview FV1000 Spectroscopic Confocal System which includes an inverted Microscope System OLYMPUS IX81 (20X, 40X (oil), 100X (oil) UIS objectives) and operates using multi Ar and HeNe lasers (458, 488, 515, 543, 633 nm). The system is also equipped with a transmitted light differential interference contract attachment, a feature that will be described in the following Section.

Images captured with the LSCM and presented in the Figures of the Experimental Chapters were often resized. Appropriate scale bars have been inserted in these Figures which take into account the applied image manipulation.
3.2.2 **Differential interference contrast microscopy (DIC)**

Three-dimensional, unstained specimens often yield poor images when viewed in brightfield illumination. This problem is solved with the use of Differential Interface Contrast (DIC) Microscopy (Figure 3.7), which allows to clearly image those challenging specimens by optical rather than chemical means.

![Figure 3.7 Schematized set-up of the DIC optics. Reprinted from: http://www.olympusmicro.com/primer/techniques/dic/dicintro.html, with permission from Olympus.](http://www.olympusmicro.com/primer/techniques/dic/dicintro.html)

The light from a lamp is passed through a polarizer located beneath the substage condenser, similarly to polarized light microscopy (114). The polarized light then goes through a Nomarski prism, which is made of two quartz wedges cemented together. Polarized light that goes through the prism is split into two beams that vibrate at 90 degrees relative to each and travel close together but in slightly different directions. The rays intersect at the front focal plane of the condenser, where they pass travelling
parallel and extremely close together with a slight path difference. Since they are vibrating perpendicular to each other, they do not cause interference. The distance between the rays is called shear and is smaller than the resolving ability of the objective. The split beams then enter and pass through the specimen where their paths are altered in accordance with the varying slope and refractive indices of the specimen. When the parallel beams enter the objective, they are focused above the rear focal plane where they enter a second modified Nomarski prism that combines the two beams at a defined distance outside the beam pairs. As a result of having traversed the specimen, the paths of the parallel beams are not of the same length (optical path difference) for different areas of the specimen. A second polarizer (analyser) is placed above the upper Nomarski beam-combining prism, which brings the two beams of different path length together into the same plane and axis, and causes interference. The light then proceeds toward the eyepiece where it can be observed as differences in intensity and colour. The result is a specimen which details appear bright on one side and darker on the other side (or with different colours). This shadow effect gives a pseudo three-dimensional appearance to the specimen, which might not be its true geometrical nature. Therefore, DIC imaging cannot be used for the measurement of actual heights and depths.

DIC optics is particularly useful in unstained samples that are truly three-dimensional, such as cells and tissues, but also artificial 3D microfabricated structures. For instance, Figure 3.8 compares an optical brightfield image of a PDMS stamp with the corresponding DIC image. The stamp is formed of a series of posts having pyramid-like shape, which can be clearly appreciated only in the DIC image.
Figure 3.8 Effect of DIC microscopy on the imaging of a three-dimensional unstained PDMS sample. (a): Optical microscopy image (60x) and (b) corresponding DIC image. Scale bar is 10 µm.
3.2.3 Scanning electron microscopy (SEM)

Scanning electron microscopy is a surface analysis technique widely used in material science. It allows monitoring the surface morphology and roughness of a flat or three-dimensional specimen, obtaining information in the nanometre range.

SEM forms micrographs of samples by focusing an electron beam of moderately high energy (0.5-50 keV) across the specimen surface (115). As a consequence, a large number of low energy (0-50 eV) secondary electrons are produced and from the outer electronic orbit of the specimen. The depth from which the secondary electrons escape the sample is generally between 5 and 50 nm. The intensity of the secondary electrons is a function of the surface orientation with respect to the incident beam and thus produces an accurate image of the specimen, with theoretical resolution up to 1 nm. These secondary electrons are influenced more by surface properties than by the atomic number of the surface atoms. Therefore, SEM cannot be used for the chemical characterization of a surface. However, the chemical information can be obtained using an additional technique attached to the SEM, which looks at the X-rays generated from the surface of the specimen by the incident probe, called Energy Dispersive X-Ray Spectroscopy (EDX or EDS). Peaks at energies characteristic of the elements within the bombarded volume can be identified and the concentrations of the elements calculated.

The first requisite for SEM imaging is for the sample to be conductive; non-conductive samples (e.g., polymers) are rendered so by coating their surface with a thin film of conductive material, most of the time gold. Coating is done under vacuum, using a sputtering machine and is irreversible.

SEM is a destructive technique for all sample types. Also, soft materials like rubbers tend to shrink during imaging, as a result of the prolonged exposure to the electron beam; thicker gold coating can reduce this effect, at the expense of resolution. Finally, samples need to be dehydrated prior imaging, for instance via freeze-drying or under vacuum. Removal of the water content can alter considerably the nature of same samples, such as hydrogels.

In the context of protein patterning, SEM is very often used for imaging the master, mold or other microstructures that are used to produce the protein micropattern. Its use
for imaging the protein micropattern itself is very limited (45), mainly for two reasons: it is a destructive means of imaging and it does not provide information on protein bioactivity.

Due to its limitations, in the work reported in this Thesis SEM was used for imaging the lithographically made masters, rubber stamps and gold-microstructures, but was not employed as a tool for imaging protein micropatterns.

3.2.4 Atomic force microscopy (AFM)

Scanning probe microscopy (SPM) (116) includes a number of techniques that use a microscopic solid probe for studying different properties of various materials. Among these, Atomic Force Microscopy (AFM) is one of the most popular due to several limitations of other SPMs. For instance, the resolution of the scanning near field optical microscopy (SNOM) is limited to around 100nm; scanning tunnelling microscopy (STM) requires operation in vacuum, while other scanning microscopes are limited to a very specific functionality. A common drawback of the SPM instruments, including the AFM, is the imaging and analysis of very small area. This is the price to pay for the very high resolution of these techniques (117).

Another important characteristic of AFM is the capability to operate in air (i.e., no vacuum needed) and in liquid environments. This is very advantageous when compared with other high-resolution techniques such as electron microscopy or optical techniques, which operate in vacuum and need special sample preparation. Finally, the atomic scale resolution offered by the AFM is difficult to achieve even using electron microscopy.

AFM can be used for imaging, probing (e.g., properties such as chemical, magnetic, and electrical) and as a tool for nano-fabrication. While the latter application of AFM (i.e., DPN) was previously discussed (Chapter 2, Section 2.5.6), the following sections will briefly discuss AFM imaging and probing capabilities.

3.2.4.1 AFM operation modes

AFM measures the interaction force (attractive or repulsive) between the probe and the surface. The solid probe is located at the end of a very flexible cantilever; an optical system detects the deflection of a laser beam that bounces off the reflective back of the
cantilever, thus reporting on cantilever fluctuations, which is proportional to the applied force. The probe is continuously moved on the surface and the cantilever deflection is constantly monitored. A feedback loop continuously changes the height of the probe on the surface in order to maintain the applied force constant. The vertical movement of the probe is recorded to create a topographic map of the surface under study.

The AFM probe tip is very sharp, with a radius of curvature in the range of tens of nanometres. If the surface under analysis is soft, the probe can penetrate it, with the risk of damaging it and of degrading the spatial resolution of the resulting micrograph. To overcome this limitation, instruments working in dynamic modes were developed. In these systems, the probe is not simply dragged on the surface but oscillated vertically with respect to the surface while it is scanned. These techniques (tapping-mode and non-contact mode) significantly reduce the probe-induced specimen damage and allow imaging soft, compressible samples, such as biomolecules and cells (117).

3.2.4.2 Lateral force microscopy (LFM)

Lateral Force Microscopy (118) studies the local interaction between the probe and the specimen. The technique maps the relative difference in the surface frictional characteristics as recorded by the cantilever-tip system while probing the surface. LFM records the lateral deflections caused by the twisting of the cantilever due to the frictional forces exerted on it parallel to the plane of the surface. Because LFM and topographic images are collected simultaneously, post-processing of these images is needed to separate one effect from the other. An improvement of the LFM method, i.e., the simultaneous measurement of normal and lateral forces, resulted in a new microscopic technique called Friction Force Microscopy (FFM).

LFM allows improving the image contrast and helps to differentiate the real image from experimental artefacts. This technique is also useful for imaging the surface physical-chemical properties as inferred from the respective surface friction. The scanning of hydrophobic surfaces using hydrophobic tips will give a lesser friction than the scanning of hydrophilic surfaces (119) due to the condensation of water molecules on the surface-tip system (120). Observed interaction forces are also small when one of the surfaces terminates with hydrophobic groups and the other terminates with polar groups, whereas
significant interactions are observed when both tip and sample surfaces terminate with hydrophilic groups due to the formation of hydrogen binding.

The post-processed image, which is obtained by subtracting the lateral force (LF) image obtained in the forward direction from the one obtained in the reversed direction, respectively, provides more information on the hydrophilic or hydrophobic nature of the sample (117). Although the friction is in general attributed to the different surface chemistry and subsequently hydrophobicity, the correlation between chemistry and hydrophobicity is unequivocal only if the surfaces contain few chemical species. Unfortunately, the information from LFM is to a large extent sample-specific, as calibration of friction forces from sample to sample is difficult and error-prone, and because minute changes in experimental conditions (e.g., humidity, tip shape) can result in important changes in friction forces. With all these qualifications, and in the absence of chemical analysis methods with sub-micrometer resolution, the LF spatial distribution can be used to infer the spatial distribution of the surface chemistry and if applicable, the local stiffness of the material (117).

3.3 PROTEIN ADSORPTION

A large portion of the research dedicated to protein patterning is directed towards the development of micropatterns (e.g., microarrays) of Immunoglobulins (IgGs), which are the proteins that were mainly studied in the work reported in this Thesis. Proteins (Rabbit IgG, Mouse IgG, Human IgG) were purchase from Sigma, received as a salt-free lyophilized powder and were reconstructed with buffer to a 2mg/mL solution. The stock solution was partitioned in aliquots and kept in the freezer. Prior an experiment, an aliquot was thawed and centrifuged (10,000 rpm, 90 seconds) to remove any precipitated protein, and then diluted to the working concentration (typically, 50 µg/mL). The buffer employed was Phosphate Buffer Saline (PBS) at the concentration of 10 mM, obtained by diluting (1:10) a stock solution (0.1 M) with deionized nanopure water (18.2 MΩ/cm). The stock solution consisted of 0.1 M KH₂PO₄/K₂HPO₄ + 0.15 M NaCl and was adjusted to pH 7.4.

Protein adsorption experiments were carried out by placing a small volume of the protein over the patterned area. Protein volume ranged between 8 to 100 µL, depending
on the size of the patterned surface. Protein adsorption was conducted at room temperature, for 30-40 minutes, inside a closed Petri dish, which had a water compartment to minimize evaporation. After adsorption, samples were copiously rinsed with buffer followed by deionized water.

3.3.1 Bioactivity retention

Protein bioactivity can be defined as the capability of molecular recognition such as the affinity interactions between primary antibodies (IgGs) and secondary antibodies (anti-IgGs).

Bioactivity retention of micropatterned IgGs was systematically investigated through secondary recognition. Bovine serum albumin (BSA) was used as a control protein in these experiments. The antibodies used for secondary recognition are the goat anti-rabbit IgG Fluorescein isothiocyanate (FITC) labelled and goat anti-mouse IgG Alexa Fluor® 546 labelled (highly cross-adsorbed), purchased from Molecular Probes (Invitrogen, Inc.) and received as 2 mg/ml solution in PBS (0.1 M PBS, 0.15 M NaCl, 5 mM NaN₃). The stock solution was partitioned in 20 µL aliquots and immediately frozen for long-term storage (maximum, 6 months). Immediately prior experiments, a stocked aliquot was diluted with PBS 10 mM to the concentration of 1 µg/mL. The excitation and emission spectra of the labelled antibodies are shown in Figure 3.9.

Additional and specific details of the methodologies employed in each work are given at the beginning of the correspondent Experimental Chapter.
Figure 3.9 Excitation and emission spectra of (a) Fluorescein (FITC) goat anti-rabbit IgG antibody (pH 8); and (b): Alexa Fluor® 546 goat anti-mouse IgG antibody (pH 7.2). Reprinted from http://www.invitrogen.com/ with permission from Invitrogen, Inc. (Australia).
4 PROTEIN IMMOBILIZATION IN LASER ABLATED BILAYERS (‘PROLAB’)

4.1 INTRODUCTION

This Chapter presents and discusses a new protein micropatterning method denominated ‘ProLAB’, i.e., protein immobilization in laser ablated bilayers. This technology allows the fabrication of profiled micro-features where proteins can be spatially immobilized with bioactivity retention. The technique combines the knowledge of an established microfabrication method- laser ablation- with a new type of substrate - a bilayer of a thin layer of metal over a thin layer of polymer. The result is a method that produces shallow micro-patterns where proteins are readily immobilized.

The bilayer substrate employed in the present work comprises a thin layer of PMMA over which a gold nanolayer is deposited, which can be removed using a UV laser. The microfabrication tool is the LaserScissors® equipped with a 337 nm pulsed laser, that, as described in the previous Chapter, can be employed as an ablative apparatus in alternative to classical, (but less encountered in biological laboratories) microfabrication tools. PMMA was chosen as the base material due to its transparency to the wavelength of the laser light used for ablation and its microfabrication characteristics, e.g., stiffness with excellent UV stability, low water absorption and high abrasion resistance. Also, the adsorption of proteins over PMMA surfaces is well documented (121), being the polymer moderately hydrophobic.

The profiled features fabricated with this method can selectively immobilize proteins, while retaining their bioactivity. Also, the ablative method allows the creation of patterns where the information (e.g., type of protein, concentration) can be encoded, for instance through a combination of vertical lines in a “bar-code” mode. Encoding the information within each pattern allows the fabrication of a multianalyte “informationally addressable” microarray. The proof of concept of this technique was published at the beginning of my candidature (see List of Publications), after which I conducted a detailed confocal study of the ‘ProLAB’ technique.
4.2 MATERIALS AND METHODS

4.2.1 Materials

Microscope glass slides and glass coverslips (0.17 mm thick, 24x24 mm) were purchased from Polysciences, Inc. Polymethylmethacrylate (PMMA), the primer 1,1,1,3,3,3-hexamethyldisilazane (HMDS) and propylene glycol methyl ether acetate (99% PGMEA) were purchased from Sigma Chemicals and used as received.

4.2.2 Substrate fabrication

Glass cover slips were sonicated in 2:1 water: ethanol solution for 30 min and washed copiously with filtered (0.2 µm) Nanopure water (18.2MΩ/cm), dried under a stream of air, and then primed with HMDS. A 4 % wt. solution of PMMA in PGMEA was spin-coated at 3000 rpm for 40 s using a Specialty Coating Systems spin coater (model P6708). The coated substrates were then soft baked at 85 °C for 60 minutes and stored in a desiccator prior to and after gold deposition. The deposition of gold (Au) was done using a sputtering SEM-coating unit E5100 (Polaron Equipment Ltd.) at 25 mA for 90 s at 0.1 Torr.

4.2.3 Laser-assisted microfabrication

Micro-laser ablation was accomplished using the Laser Scissors® Workstation (module 337/120, Cell Robotics, Inc., U.S.A.) mounted on a Nikon Eclipse TE300 inverted microscope and coupled with a computer controlled, pulsed nitrogen laser (VSL-337 Nitrogen Laser, Laser science, Inc., U.S.A.). The laser emits at 337 nm, with a maximum power output of 2 mW, maximum intensity of 120 µJ/pulse, and pulse duration of 3-4 ns. The laser beam is focused directly on the gold layer through the microscope objective lenses (40X). Ablation is conducted at a rate of 20 pulse/sec and a speed of 20µm/sec, at ambient temperature (22°C) and a relative humidity of 45%.

4.2.4 Protein adsorption

IgGs (Rabbit IgG, Mouse IgG and Human IgG) were used at the working concentration of 50 µg/mL in PBS. The secondary antibody anti-rabbit IgG-FITC labelled was used at
the concentration of 1 µg/mL. BSA was used as a control protein at the concentration of 50 µg/mL.

Laser-ablated regions were incubated with an 8 µL droplet of 50 µg/mL protein solution. After 30 minutes, the samples were rinsed with PBS (3x10 mL) and with deionized water (3x10 mL), and dried under a stream of air. For secondary antibody recognition experiments, firstly, the unlabeled primary antibody was adsorbed and after rinsing and drying, the secondary labelled antibody was incubated for 30 minutes. The sample was then washed as described above, air dried and imaged.

4.2.5 Characterization

4.2.5.1 UV/Vis

Transmission of PMMA and Au in the 200-800 nm range was obtained using two 1mm-tick quartz slides (SPI Supplies, Inc.) coated with PMMA and gold, respectively. The thin polymer layer was obtained by spin coating the PMMA solution (4% wt. in PGMEA) over the quartz slide, followed by baking at 85°C for 60 minutes. The thin gold layer was obtained by sputtering the quartz slide with gold as described in Section 4.2.2. Transmission measurements were determined on a Cary 50 UV-Vis spectrophotometer (Varian, Inc.).

4.2.5.2 SEM

Ablated microstructures were imaged with SEM using a JEOL microscope (JSM840) at 15 kev. Elemental identification of ablated gold was performed using energy dispersive X-ray (EDX) analysis.

4.2.5.3 AFM

Atomic Force Microscopy was carried out on a Topometrix Explorer system (ThermoMicroscopes) in the normal contact mode. Several scanners were used in order to cover the scales of lateral topographical and chemical differentiation; the fields-of-view ranged from 100x100 µm down to 8x8 µm. The analyses were carried out under air-ambient conditions (temperature of 22 °C and 45% relative humidity). Pyramidal-tipped, silicon nitride cantilevers with a spring constant of 0.032 N/m were used. As the
tip is scanned across the surface, the lateral force acting on the tip manifests itself through a torsional deformation of the lever, which is sensed by the difference signal on the Left-Right signal on the quadrant detector. The difference signal can be plotted as a function of x-y location in the topographical field of view, and the resulting friction force image can then be correlated directly with the topographical image. In this study, the lateral force imaging was performed simultaneously with topographical imaging in both forward and reverse scan directions.

4.2.5.4 Laser scanning confocal microscopy (LSCM)

Microablated structures were imaged with the LSCM before and after the adsorption of labelled proteins. In both cases, imaging was done with the 40X objective, using the 488 nm laser line (6% power), Channel 1 (Excitation 488 nm laser, Emission 520-540) and same acquisition settings (in terms of scan speed, voltage and gain). In addition, a dichroic mirror (SDM 510) was used to cut away the strong signal arising from the light reflected on the gold surface. That way, only the fluorescence emission arising from the dye was detected.

Microablated patterns with adsorbed proteins were also imaged at higher magnification using the 100X oil objective, together with the zoom-in capability of the microscope. In this case, the settings were changed (laser power 12%) to obtain the best images in terms of contrast and resolution.

4.2.5.5 Image analysis

The intensity profiles and intensity surface plots were generated using the LSCM software (FV10-ASW Version 1.3, Olympus) or Image Pro® Plus (Version 5.0, MediaCybernetics®), respectively, using the original confocal images, without any modification. Image Pro® Plus was also used to measure the dimensions of the microchannels, using a picture of a ruler (captured with the corresponding magnification) for calibrating the image. For a given measure, 10 values were collected and averaged. The same software was used to measure the gold nanospheres using a calibrated SEM image.

The intensity integral of fluorescent nanospheres was calculated using the in-built capability of the FV10-ASW software by defining a ROI for each nanosphere.
4.3 RESULTS AND DISCUSSION

4.3.1 Overview of the proLAB technique

Figure 4.1 is a schematic illustration of the protein patterning strategy employed in this work.

The gold/PMMA (Au/PMMA) bilayer substrate is fabricated by spin coating a PMMA solution over a clean glass substrate, which, after solvent evaporation, leaves a thin polymer film. A thin layer of gold is subsequently sputtered over the PMMA surface, and then micro-ablated with the 337 nm pulsed laser of the LaserScissors®, leaving micro-areas where proteins can be adsorbed. Since proteins tend to adsorb fairly strong to bare Au surfaces (122), to achieve highly selective protein patterning it is necessary to “deactivate” the Au substrate against protein adsorption, which is done by coating the Au/PMMA bilayer with BSA 1% w/v before the laser ablation step.
4.3.2 Laser-ablation of the Au/PMMA bilayer

The transmission spectra of a thin film of gold and of a thin film of PMMA are shown in Figure 4.2; the spectra confirm that the adsorbing material at 337 nm is the gold. As a consequence, focusing the beam of the UV laser over the metallic surface readily ablates the gold revealing the underlying polymer.

![Figure 4.2](image)

**Figure 4.2** Transmission spectra of a thin layer of gold (---) and PMMA (-----) deposited on quartz.

The thickness of the gold layer is an important parameter of the ablative process, with gold layers over 50 nm not easily ablated, and layers of 100 nm totally resistant to ablation. Moving the stage while performing the ablation allows the generation of different geometries. Here, lines were chosen as the structures of interest, being a bar-coded pattern the final targeted platform. The quasi-continuous ablation of the gold film can be achieved with a rate of 20 pulse/s and a speed of the microscope stage of 20 µm/sec. Higher stage velocities result in discontinue ablation. Changing the laser power during the ablation process creates channels with different widths, as shown in Figure 4.3, which is a representative bright field image of a set of six microchannels formed by ablating a substrate using 100, 75, 50, 25, 10 and 5% (from right to left, respectively) of the laser power. Further reduction of laser power results in lines poorly ablated.
Figure 4.3 LSCM (40X) transmission light image of a series of microchannels ablated with varying laser powers. Scale bar is 10 µm.
4.3.2.1 Characterization of ablated microchannels

4.3.2.1.1 SEM

Figure 4.4 shows SEM imaging of the ablated channels presented in Figure 4.3. Increasing the laser power during ablation leads to a broadening of the ablated lines, with dimensions summarized in Figure 4.5. For the set writing parameters (i.e., objective magnification, stage speed and pulse rate) the smallest channel that can be fabricated is 2.5 µm wide when 5% of the laser power is used, whereas when using the full power of the laser, channels about 9 µm wide are fabricated.

![SEM micrographs of six microchannels ablated an Au/PMMA bilayer substrate, fabricating varying the laser power.](image)

**Figure 4.4** SEM micrographs of six microchannels ablated an Au/PMMA bilayer substrate, fabricating varying the laser power.
The SEM images clearly show nanoparticles of around the ablated area, and along the edges of all ablated microchannels. EDX analysis of these particles (Figure 4.6) shows that these are gold particles.

**Figure 4.5** Width of the ablated microchannels as a function of the laser power employed during ablation.

**Figure 4.6** EDX of gold nanosphere performed on a nanosphere outside the ablated microchannel.
Above a laser power of 25%, the high amount of debris deposited results in uneven edges, leading to channels with variable width. Quantitatively, this is reflected in higher standard deviations for the corresponding widths measurements in Figure 4.5.

4.3.2.1.2 AFM

AFM analysis of the same set of ablated microchannels is presented in Figure 4.7. In all samples, the edges of the microchannel are elevated above the level of the gold layer. Also, the topographical analysis reveals the formation of a shallow nanochannel inside the centre of the lines ablated with a laser power above 25%. The depth of this inner nanochannel is about 30 nm inside the wider line (100% of laser power), about 15 nm inside the line ablated with 50% of the laser power and approaches zero when ablation is performed using 25% of the laser power.
**Figure 4.7** AFM (topography) of three microchannels ablated using (a) 25%, (b) 50% and (c) 100% of the total laser power during micro-ablation. Depth of the core nanochannel is highlighted in each line analysis.
4.3.2.1.3 LFM

In Chapter 3 (Section 3.2.4.2) it was discussed how lateral force microscopy can be used, albeit with caution, to infer information regarding the hydrophobic or hydrophilic nature of a surface. Figure 4.8 shows a microchannel ablated with 100% of the laser power and analysed with LFM, together with the corresponding topographic image.

The LFM micrograph if Figure 4.8 shows three distinct regions in terms lateral force: (1) the inner area of the channel, which shows the most negative force values; (2) the edges of the channel, which show the higher force values; and (3) the surface between the core and the edged of the microchannel, with intermediate values. These results suggest that, compared to the outer gold surface, the core of the microchannel is hydrophobic, whereas the edges are hydrophilic.

**Figure 4.8** AFM micrographs (topography and later force) of microchannel ablated using 100% of the laser power.
The AFM and LFM analysis can be summarized as follows:

1. Laser ablation of the bilayer generates a microchannel that, when a laser power higher than 25% is used, shows an inner shallow nanochannel, which depth reaches a maximum of about 30 nm when 100% of the laser power is used during the ablation;

2. The edges of the microchannel are ~100 nm elevated above the level of the gold layer.

3. Together with a distinct nanotopography, the LFM analysis of the microchannel ablated with higher power suggests that this has a hydrophobic core, whereas its edges are hydrophilic.

4.3.2.1.4 LCSM

Figure 4.9 (a) is a LSCM (fluorescence) image of the set of lines ablated with 5, 10, 25, 50, 75 and 100% of the total laser power, respectively. The image was acquired using the 40X objective and the 488 nm laser line. A low fluorescent signal (S/N =2.2 in the line ablated using 100% of the laser power) was detected inside the microchannels. In order to visualize it, the contrast and brightness of the image was enhanced. A gold-coated slide (without the PMMA layer) was used as a control, where channels were ablated and imaged under identical conditions. No fluorescent emission was detected for this control, even when enhancing the image. Also, confocal imaging of a control PMMA coated slide (without Au coating) revealed that the polymer is not fluorescent. Therefore, the fluorescent signal in Figure 4.9 cannot be attributed to PMMA or gold but to some of degradation products produced during ablation.
Figure 4.9 (a) LSCM (fluorescence) image of the microchannels ablated with different laser power (indicated in brackets); (b) corresponding intensity profile of the fluorescence emission within the ablate microchannel (highlighted with orange dotted lines). Scale bar in (a) is 10 µm.
For the line ablated with 25% of the laser power or less, the associated intensity profile is homogeneous throughout the channel (Figure 4.9 (b): C). When higher energies are used, though, the intensity profiles of the channels clearly show a drop in fluorescence intensity in the central area of the channel. This effect is most pronounced for the line ablated with 100% (Figure 4.9 (b): F) of the laser power, and reaches a minimum for the line ablated with 25% of the laser power. When lower laser powers are used (below 25%) this effect is absent (Figure 4.9 (b): A and B).

In summary, these results show that during the ablation process, PMMA experiences some form of chemical degradation, which leads to by-products that are mildly fluorescent. When ablating with a laser power of 25% or above, LSCM imaging of the generated microchannels reveals a fine structure, with an inner area considerably less fluorescent. These results are consistent with the AFM and LFM analysis, which have shown the presence of a nanochannel with distinct physiochemical and topographical characteristics inside the line ablated with 100% of the laser power.

**4.3.2.2 Description of the laser-ablative process**

The ablation of the bilayer substrate involves the interaction of the laser with two materials, namely gold and PMMA. Although gold is the adsorbing material, the fate of the underlying polymer during the ablative process needs to be considered as well. Therefore, both interactions are discussed first individually, and then in combination.

**4.3.2.2.1 Laser interaction with gold**

The precise physiochemical mechanisms involved in thin film metal ablation depend both on laser parameters (e.g., energy and fluence) and ambient conditions (e.g., temperature and pressure). Compared with the laser ablation of bulk metals, thin film ablation with pulsed lasers is far more complicated (123). This arises from the poor thermal conductivity of the underlying substrate (a dielectric substrate in microelectronic applications, PMMA in this case); therefore the laser energy is more confined within the metallic film, causing longer melting process. It has been observed (123) that the long period of the molten state in the film is expected to promote both vaporization (i.e., atomic removal of particles from the surface at elevated temperatures during and after laser irradiation) and hydrodynamic development (i.e., vaporized
atomic particles interact with the ambient gas forming a gold vapour cloud, resulting in a local pressure near the film surface, which enhances hydrodynamic instabilities and results in the ablation of liquid in the form of droplets (123)). A hydrodynamic mechanism was proposed for the excimer laser ablation of a gold thin film (KrF laser, 248 nm) deposited on a quartz crystal microbalance (123).

In addition, a recent work (124) shows how the interaction of a laser (532 nm) with gold microstructures, in certain conditions, results in the formation of gold nanospheres. When fluences leading to gold melting are used, a dewetting process is started and the liquid then contracts towards a nanosphere. In the cited work, when higher laser fluences were used, detachment of the droplets was also observed in what the authors described a ‘jumping nanodroplets’ phenomena.

In conclusion, these works suggest that the interaction of the laser with the gold layer can induce gold vaporization, melting, or a combination of these. In both cases, ablation products in the form of re-solidified gold are expected, with the possible formation of highly-symmetric nanostructures, such as nanospheres.

4.3.2.2.2 Laser interaction with PMMA

According to the literature (125), the first significant adsorption band of PMMA is at 220-230 nm corresponding to adsorption of the ester group (COOCH$_3$) (125). Therefore the ablation of PMMA is normally performed with high-energy lasers (e.g., 193 nm); alternatively, if a laser with lower energy is used (e.g., 308 nm) it is necessary to dope the polymer with organic dyes that adsorb in this region.

When using lasers having a wavelength below 260 nm, the principle mechanism of decomposition appears to be photochemical (34). At UV wavelengths above 260 nm, the ablation mechanism becomes more complex, comprising photochemical and photothermal decomposition, and at wavelengths above 308 nm the mechanisms becomes entirely photothermal (125), (34). The products of the laser ablation of PMMA at 193 nm and 248 nm include the monomer, MMA (methyl methacrylate), CO$_2$ and low molecular weight fragments of PMMA (126). In contrast, when ablating at lower energies (308 nm) the only ablation products are the monomer and, in some cases, its dimer (127), consistent with a thermal decomposition of PMMA (128). Also, it has
been reported that the heat generated during the interaction of PMMA with a 308 nm laser can cause the polymer to melt (125). In the present work, PMMA is exposed to a 337 nm laser; therefore thermal decomposition as well as melting is expected. However, cited works used high-energy continuous lasers to study the decomposition of commercial sheets of PMMA (about 650 µm thick); here, the situation is very different, since the polymer is deposited as a very thin layer, over which a thin layer of gold is deposited. Also, in the present work the laser used is pulsed, not continuous. As it will be discussed in the sections that follow, the gold layer on top of the PMMA layer should adsorb most of the laser energy, but because of the very thin nature of the bilayer substrate, local heat is expected to alter the surface proprieties of PMMA through thermal degradation and/or melting.

4.3.2.2.3 Laser interaction with the Au/PMMA bilayer

The ablation of the bilayer should be the combination of the effects of the laser interaction with both materials, Au and PMMA. In light of the characterization results for the ablated microchannels and literature information, the following qualitatively description of the ablative process is proposed.

The intensity profile of a laser is a Gaussian distribution of the energies (34); therefore the ablation process is expected to vaporize the gold at the centre of the channel more effectively than at edges of the channel. Laser exposure concurrently causes the overheating of the top layer of the underlying PMMA to a point where the polymer is melted and chemical reactions start to occur. The LFM analysis of the ablated microchannels (Figure 4.8) shows that these present a lateral variation of hydrophobicity, with the edges of the channels being the most hydrophilic and the centre the most hydrophobic. This variation of surface chemistry can be attributed to the lateral distribution of the ablation energy, which translates in different energies delivered to the polymer, and subsequently different surface chemistries. The expected reactions would be, in the order of increasing pyrolysis temperature, i.e., from the edges towards the centre, (i) the termination of the side ester groups at one of the C-O bonds, resulting in a more hydrophilic material, (ii) depolymerization of the main chain, preserving the same hydrophobicity, and, if the process is quick enough (i.e., higher fluences), (iii) the breaking of the side bonds, resulting in a more hydrophobic material,
possibly due to the formation of aliphatic chains. Therefore, it is possible that when ablating the Au/PMMA bilayer with the full power of the laser, three regions are formed in the micro-channel, i.e., a hydrophobic core, a hydrophilic rim and an area with intermediate hydrophobicity. This model is consistent with the AFM topographic and LF results.

The hydrophilic edge of the microchannels also includes gold nanoparticles, as shown from the SEM results, which originate form the re-solidification of vaporised and/or melted gold. Gold nanoparticles are also found outside and, occasionally, inside the channel, often in the form of regular nanospheres, which probably arise from a ‘jumping nanodroplets’ phenomena similar to the one described in the literature (124).

Ideally, a micro-chemical analysis (e.g., XPS) should be performed to chemically identify the ablative products in each region and fully characterize the ablated microchannel. This task is extremely hard to accomplish, due to the limited size of the area and the possible presence of numerous ablative products. However LSCM results previously described (Section 4.3.2.1.3) provide some valuable information regarding the fluorescence of the ablated area, which gives indirect information on its physiochemical properties. Compared with un-irradiated PMMA, ablated areas are more fluorescent (Figure 4.9). This increased fluorescence is probably due to conjugated product arising from PMMA thermal degradation. As previously mentioned, the formation of methylmethacrylate (MMA) and its dimmer has been reported during PMMA thermal degradation (128), which are both conjugated species. When ablation is done using the full power of the laser, the resulting microchannel shows an inner area significantly less fluorescent. This result is in accordance with the hypothesis that, in those conditions, the thermal degradation of PMMA inside the microchannel reaches a maximum, with formation of aliphatic, hydrophobic products, inherently non-fluorescent.

4.3.3 Protein adsorption

Laser-ablated microchannels were used for the spatial immobilization of proteins, in particular, immunoglobulines. In these experiments, a BSA-coated Au/PMMA substrate was employed, microablatted as described above to form channels where proteins were immobilized via spontaneous adsorption.
Figure 4.10 illustrates the adsorption of anti-rabbit IgG-FITC labelled inside microchannels ablated with different laser powers. As shown by the correspondent intensity surface plots in Figure 4.11, when using laser powers above 25%, proteins adsorb preferentially along the inner area of the channel.

**Figure 4.10** LSCM (fluorescence) images (100X) showing the adsorption of a labelled rabbit IgG inside microchannels ablated with varying laser power (indicated in brackets). Scale bar in (F) is 10 µm and applies to all images.
The fluorescence trend in Figure 4.10 is opposite to the one in Figure 4.9 (LSCM imaging of the microchannels before protein adsorption). For microchannels ablated using the full power of the laser (Figure 4.10 (F)), a fluorescent nanochannel is clearly visible, indicating a preferential adsorption in this region. This effect is evident up to the channel ‘C’, which was ablated with 25% of the laser power. Chapter 2 discussed the high sensitivity of proteins to surface properties, and how hydrophobic surfaces
generally promote protein adsorption. Therefore these results confirm the different physiochemical characteristics within the inner area of the ablated channel, which are ‘sensed’ by the protein and induce a variation in protein adsorption. These results, together with the LFM analysis previously discussed, indicate that when ablating using more than a 25% of the total laser power, a hydrophobic core is generated in the channel, where protein preferentially adsorb. At lower laser powers, the ‘hydrophobic core’ is no longer formed; proteins still adsorb inside the ablated microchannel but proportionally less (Figure 4.11, C), down to a point where most adsorption is on the edges of the microchannel (Figure 4.11, A). The latter phenomena might appear in contradiction with the general knowledge that hydrophilic surfaces repel proteins. The chemical and topographical nature of the edges of the microchannels, though, is probably extremely complex. First, if carboxyl groups are present in this area (as a result of PMMA de-esterification) they could be de-protonated. The resulting charged surface can have an unexpected effect on protein adsorption, as also reported in other works (49). Secondly, the roughness of the edges of the microchannels probably plays a role on protein adsorption, for instance increased specific area. As it will be discussed in later in the Chapter (Section 4.3.3.2), this effect is not easy to quantify.

4.3.3.1 *Bioactivity of patterned IgGs: specific antibody recognition*

To test the bioactivity retention of an adsorbed protein, a specific antibody recognition experiment was performed. Rabbit IgG (un-labelled) was adsorbed over an ablated area of a freshly prepared substrate. BSA was adsorbed over a second ablated area, as a control, and a third area was kept free from protein adsorption. Each area was ablated with 50% of the laser power. All proteins were incubated simultaneously inside a humid-saturated chamber at room temperature. After repeated rinsing with buffer and water, the secondary antibody anti-rabbit IgG FITC- labelled was deposited over the entire substrate. The sample was then rinsed and air-dried and immediately visualized with the 488 nm laser beam of the LSCM. Each area was imaged under the same acquisition parameters using the 40X objective. Figure 4.12 is a composite image of the control area (A) and the two protein-adsorbed areas (B: BSA; C: Rabbit IgG). The intensity profile corresponding with each image clearly demonstrates how the anti-rabbit IgG specifically recognized the corresponding IgG lines. The BSA control area shows an increase signal compared to the control (A), which is probably due to
unspecific adsorption of the second antibody over unoccupied sites inside the microchannel. Nevertheless, the signal in C is significantly higher then the signal in both controls (S/N=7.6). Hence, specificity antibody recognition is confirmed, which, in turn, is an indication of bioactivity retention of the adsorbed Rabbit IgG.

Figure 4.12 LSCM images showing specific antibody recognition in microchannels ablated using 50% of the total laser power. A: control, an ablated microchannel without proteins; B: second control, a microchannel with adsorbed BSA; and C: a microchannel with adsorbed rabbit IgG. B and C were subsequently reacted with anti-rabbit IgG FITC-labelled. Scale bar in (C) is 10 µm and applies to all images.
4.3.3.2 Effect of surface nanotopography on protein adsorption

The ablation of the Au/PMMA bilayer generates patterns with complex nanotopography: when high laser power are used, a nanochannel 30 nm deep is formed in the middle area of the ablated channel, with concomitant formation of gold nanoparticles along the channels’ edges; when low (less then 25%) laser power are used, the inner nanochannel is not formed but nanoparticles are still deposited along the edges of the ablated channel. The question therefore arises of if and how these nanotopographies influence protein adsorption.

As already discussed (Section 4.3.2.2.3), it was not possible to chemically characterize the nanochannel formed in the core of the micro-ablated area. However, qualitative evidence suggests that it comprises various thermal degradation products of PMMA. Therefore, inside the microchannel, it is not possible to interpret the adsorption results solely as a function of surface nanotopography, i.e., surface chemistry needs to be considered as well. Also, the nanotopography at the edges of the nanochannels is very complex. Although EDX results show that gold particles are present in this area, it is likely that they are mixed with PMMA degradation products. Also, the size and shape of these particles are very variable in size and shape. These characteristics make this area unsuitable for studying the relationship between nanotopography and protein adsorption. In contrast with the edges of the microchannel, regular gold nanospheres are often encountered outside the ablated region. As previously mentioned, literature (124) suggests that these spherical gold nanoparticles are generated from the re-solidification of melted gold. A graphic software can be used to measure the dimensions of these microspheres from a calibrated SEM image. After adsorption of a labelled protein, the software interfacing with confocal can be used to calculate the fluorescence integral of each nanosphere. The relationship between the area of the nanospheres and their fluorescence emission can then be analysed. To study this relationship, the sample reported in Figure 4.12, (which was used for demonstrating bioactivity retention of adsorbed IgG) was used. The fluorescent signal is that of the secondary antibody anti-rabbit IgG-FITC labelled attached to the primary antibody rabbit IgG, attached via biorecognition rather then unspecific adsorption, as previously demonstrated (Section 4.3.3.1, Figure 4.12).
Figure 4.13 (a) shows the SEM image and the corresponding LSCM image of a large area outside a microablated channel from which the dimensions of a number of particles (30 in total) were obtained using Image Pro Plus®. The LSCM image in Figure 4.13 was acquired using the 100X objective and the acquisition settings adjusted so to have a lower fluorescent signal compared to Figure 4.12, in order to visualize small variations of fluorescence intensity. The SEM image was used as a guide to correctly identify the nanospheres, and therefore select specific ROIs in the LSCM image. For example, Figure 4.13 (b) shows the LSCM transmission and fluorescence images, together with the corresponding SEM micrographs of a series of nanospheres that were used for this calculation. The fluorescence integral of each selected particle was then calculated using the in-built capability of the software that interfaces with the confocal microscope.

Before protein adsorption, the gold nanospheres are not fluorescent, as revealed from an LSCM analysis of the control sample at high magnification (as well from the control 9a) in Figure 4.12). Also, as discussed in Section 4.2.5.3, imaging was done using a dichroic mirror before the emission channel to eliminate any light reflected by the gold. Therefore, the fluorescence in the LSCM image in Figure 4.13 can be safely attributed to the emission of the dye in the labelled protein (anti-rabbit IgG-FITC).
Figure 4.13 (a): SEM and LSCM images that were employed for correlating gold nanoparticles size with fluorescence density; (b) transmission, fluorescence and SEM images of a series of particles.
When the integral of the intensity curve associated with each particle was plotted against the correspondent nanoparticle area, no relationship between the two was obtained, i.e., the intensity density appears independent of nanoparticle size (Figure 4.14). Therefore, these results indicate that nanoparticles sizes in the range of 170-400 nm do not influence the adsorption of the protein here investigated (anti-rabbit IgG). This result is not surprising when comparing the size of the IgG protein (~20x25x4 nm) (80) with the smallest gold nanoparticle in Figure 4.14 (i.e., ~170 nm). It was previously observed that it is possible that nanoparticles can influence protein absorption only when their average length scale is of the same size or smaller than the dimension of the protein under investigation.

![Figure 4.14](image.png)

**Figure 4.14** Graph showing the lack of correlation between gold nanoparticle area and fluorescence intensity integral

Comparison of these results with literature information is not trivial, since the effect of surface nanotopography on protein adsorption is not well documented, and most work published deals with the influence of nanotopography on cell-adhesion, therefore looking at proteins involved in the adhesion process, such as collagen (105)). Proteins involved in cell adhesion, though, greatly differ in structure from the types of proteins investigated here, i.e., immunoglobulins. Few recent works have investigated the
influence of globulin protein adsorption to surface nanotopography, which are more relevant to this work, since immunoglobulins are a sub-class of γ-globulins. Müller and co-workers (129) have used nanometre-scale roughness in the form of germanium nanopyramids to study the effect of these on the adsorption of BSA and γ-globulin (129). The adsorption of both proteins increased as the roughness of the surface did, which varied between 2.7 and 10 nm, but specific antibody recognition studies for γ-globulin demonstrated loss of protein activity on nanometre-scale rough surfaces. The authors concluded that these surfaces gave rise to a strong interaction of γ-globulin, changing the protein conformation. Similarly, another work (130) reported the adsorption of protein A (a protein that has a high affinity for IgGs) on nanostructures generated on silicon in the form of 30 nm lines with irregular spacing of about 40 nm. No differences in adsorption behaviour of protein A on nanostructured or neat surfaces was found, but once again the interaction with the nanostructured surface caused the protein to lose its bioactivity. Finally, Pallandre and co-workers (131) recently reported the effect of surface nanostrips on the orientation of P.69 pertactin, a compact globular antigen. Nanostrips of alkylsilane on a background of PEO having 20, 35, 55, 90 and 170 nm width were used. Here, the protein was found to orient differently depending on the width of the stripe, assuming a side-on orientation over the narrower stripes (width< 50 nm). The bioactivity of the protein after adsorption over the nanostrips, though, was not investigated.

Taken together these results confirm what was discussed at the end of Chapter 2, i.e., it is not possible to generalize the effects of protein interaction with nanometre-scale roughness. The type of protein used, the chemical nature of the surface, and the nanotopography characteristics (size and geometry) contribute to the outcome of protein-material interaction. Overall, it appears that nanometre-scale roughness can alter protein adsorption and orientation, but this can sometimes be associated with loss of protein bioactivity. Here, protein bioactivity is preserved as indicated by the retained affinity of the primary antibody (Rabbit IgG) for its labelled secondary antibody, both inside the microchannel and along its edges, where nanoparticles are abundant. Analysis of the fluorescent signal outside the micro-channel versus particle size indicates that the fluorescence density is independent of size of the nanoparticles. Fluorescence microscopy cannot be used as a tool for protein absolute quantification but, relatively,
these results suggest that the adsorption of Rabbit IgG is not influenced by the nanoparticles sizes. However, this result cannot be generalized to the effect that the nanofeatures along the micro-channel edges have on protein adsorption. The particles considered in Figure 4.13 are spherical, the smallest being 170 nm; in contrast, along the edges of the microchannels, smaller, irregular nanoparticles are formed, illustrated in Figure 4.15, which are likely to have a different effect on protein adsorption. This effect could not be fully analysed in this work and therefore leaves an open question that needs to be addressed in further studies.

Figure 4.15 SEM micrographs of an ablated microchannel showing complex nanotopography along its edges
4.3.4 A multianalyte bar-coded microassay platform

One of the advantages of a direct writing approach as the one used in this work is that it allows creating patterns for protein immobilization where the information (e.g. type of antibody, concentration) can be encoded, for instance through a combination of vertical lines in a “bar-code” mode. This allows the development of a microassay platform that is “informationally addressable” rather than only “spatially addressable”, like in the classical microarrays. Figure 4.16 schematizes the transition between a classical microarray to a bar coded microassay platform.

![Figure 4.16](image)

**Figure 4.16** Conceptual transition from computer-spotted microarrays (a) and bar-coded microarrays (b). Images not to scale.

A bar-coded multianalyte microassay is presented in Figure 4.17. Four different regions were ablated under the same conditions (40X objective, 20 pps, 20 µm/sec stage speed) using 5% of the total laser power in order to fabricate the smallest array. Each area was made of four lines 40 µm long, which were organized in four different bar-codes. Three different IgGs, namely Human IgG (A), Mouse IgG (B), Rabbit IgG (C) were adsorbed in three separate regions, and BSA (D) was adsorbed in a fourth area as a control. Protein adsorption was performed simultaneously by placing a small droplet of the protein solution over each area. After extensive washing, the whole sample was incubated with BSA (1% w/v) to block any unoccupied sites within the micro-areas. Following washing and drying, anti-rabbit IgG FITC labelled was incubated over the...
entire sample. Specific antibody recognition (C) occurred, as illustrated in Figure 4.19, with excellent specificity.

Figure 4.17 A multianalyte bar-coded microassay. Bar coded micropatterns were adsorbed with unlabelled (A) Human IgG, (B) Mouse IgG, (C) Rabbit IgG and (D) BSA, respectively. The patterns were then reacted with anti-rabbit IgG FITC-labelled and imaged at the LSCM (40X). Scale bar is 10 µm.

The fluorescence signal in Figure 4.17 (C) is stronger along the edges of the microchannels. This effect was previously reported and described (Section 4.3.3) and is probably due to physiochemical properties of the microchannel ablated with the lowest energy. To generate patterns with higher and more uniform S/N (as in Figure 4.12), a higher laser power should be used (e.g., 50%) with the consequence of larger features. Therefore, although the smallest microchannel that can be fabricated with the ‘proLAB’ technique is about 3 µm, when employed for protein patterning, realistically, this minimal dimension is about 6 µm.
4.4 CONCLUSIONS

UV laser ablation, together with a specific bilayer substrate (Au/PMMA), can be successfully used for fabricating shallow microstructures where proteins can be selectively immobilized with bioactivity retention. In this specific work, microchannels were chosen as the structure of interest with the aim of arranging them in ‘bar-codes’, so to create an ‘information-addressable’ microarray. This platform was fabricated and its application to specific antigen binding demonstrated. Three different IgGs (namely, mouse IgG, human IgG and rabbit IgG) were first immobilized; the bar-coded array was then exposed to a solution of labelled anti-rabbit IgG, when selective recognition of the rabbit IgG occurred.

Laser ablation of the bilayer substrate can be done using different percentages of laser power. In this work, the different microchannels analysed were produced using 100, 75, 50, 25, 10 and 5% of the total laser power. SEM, AFM, LFM and LSCM analysis showed that changing the power of the laser during ablation has an effect on the surface properties of the microchannel, both in terms of nanotopography and hydrophobicity. When ablating using the laser at its full power, a 30 nm-deep hydrophobic core is created in the middle of the channel. This effect is reduced when using lower energies, to a point where this inner nanochannel is no longer formed. LSCM analysis of this series of lines revealed that the ‘core nanochannel’ is non-fluorescent. This information, together with the knowledge from LFM that this nanochannel is hydrophobic, suggests that it is formed of aliphatic product arising from the thermal degradation of PMMA. In all samples analysed, a hydrophilic rim is formed at their edges, possibly containing PMMA de-esterification products. In addition, SEM and EDX show that gold nanoparticles are deposited along the edges as well as outside of the ablated area, probably arising from the quick re-solidification of melted gold.

This complex level of nanotopography and surface chemistry makes the full characterization of the microablated channels challenging. However, when used for protein adsorption, proteins appear to ‘sense’ the distinct topographical and chemical properties of the ablated channels. When higher laser powers are used (up to 50% of the total), protein adsorption is preferential inside the micro-ablated area, which corresponds to the hydrophobic region of the channel. When lower laser powers are
used, the hydrophobic core is no longer formed and, consequently, the adsorption inside the channels is relatively lower. In all cases, proteins adsorb along the edges of the channel which, according to LFM, are hydrophilic. Numerous factors could be involved in this result, such as the presence of charged groups and/or surface roughness. However, the very complex chemical and topographical nature of the edges of the microchannels makes this area unsuitable for studying the relationship between protein adsorption and nanotopography. These types of study need to use fairly simple topographies with known chemistry, regular shapes and a small range of sizes.

In contrast with the edges of the microchannels, spherical nanoparticles are encountered outside the ablated area, ranging from 170 nm to 400 nm. The formation of similar, spherical nanoparticles during laser ablation of gold is consistent with literature information (124). Therefore, the influence of nanotopography on the adsorption of a labelled protein was investigated in this area. The sample used in this work is the outer area of a micro-ablated channel used for a recognition experiment, i.e., where rabbit IgG (unlabeled) was first adsorbed and then reacted with anti-rabbit IgG FITC labelled. A control sample was also used to confirm that biorecognition, rather than unspecific adsorption, had occurred. To study the influence of topography on relative protein adsorption, a calibrated SEM micrograph and the correspondent LSCM image after protein adsorption of this sample were employed and 30 regions of interest (i.e., 30 nanoparticles) were selected. The fluorescent signal in the LSCM image was safely attributed to the FITC dye by studying a control sample having numerous gold nanoparticles, which were non-fluorescent.

When the intensity integral calculated by the confocal software was plotted against the area of the particles, no relationship was found, indicating that the fluorescence density (indirectly related to the protein density) is independent on the area of the particles. This result is consistent with other studies which have shown that protein adsorption can be influenced by nanotopographies having dimensions comparable with that of the protein under study, for instance altering its orientation on a surface. This is not the case here since the nanoparticles are at least one order of magnitude larger than the protein. However, nanotopography often has a detrimental effect on protein bioactivity. Here, nanotopography, as indirectly estimated by relative fluorescence, did not influence protein adsorption on the gold nanospheres, but their bioactivity was maintained.
In summary, the UV laser ablation of the Au/PMMA bilayer allows fabricating patterns suitable for the spatial immobilization of proteins with bioactivity retention. This method (denominated ‘proLAB’) can be used to create microarrays of encoded patterns, therefore improving the read-out of the array. The laser ablation conditions employed during the fabrication process have an effect on the surface chemistry (in terms of hydrophobicity) and topography of the resulting pattern. To assess protein adsorption solely as a function of nanotopography, regular nanofeatures with known chemistry need to be employed. Here, spherical nanoparticles are generated outside the ablated area. EDX analysis indicates that these are gold nanospheres. The influence of these on protein adsorption was therefore studied, using the relative fluorescence emission as an indirect indication of protein adsorption. For nanospheres in the range of 170 nm to 400 nm, fluorescence density was found independent of the area of the nanosphere, but protein bioactivity was retained.
5 MICROCONTACT PRINTING PROTEINS TRAPPING AIR (‘μCPTA’)

5.1 INTRODUCTION

Soft-lithography was originally developed as an alternative microfabrication technology for bio-applications. As discussed in Chapter 2, this technology comprises a series of methods that use a biocompatible stamp for patterning biological substances. Among these methods, thanks to its simplicity and repeatability, μCP has received growing attention in the last decade as a tool for the spatial immobilization of biomolecules and cells. The use of μCP for patterning biomolecules, in particular proteins, was extensively reviewed in Chapter 2 (Section 2.5.1).

The polymer commonly used in μCP is Sylgard 184 PDMS, which has a density of \( \sim 1 \) g/cm\(^3\) and a Young’s modulus of \( \sim 3 \) MPa (111). In conventional μCP, the three-dimensional pattern on the stamp must be designed to withstand the forces involved in the printing process. The relief pattern on a stamp can be considered as a series of features elevated from a base plan. Due to the relatively low Young’s modulus of PDMS, only a small subset of the relief features created by replicating a silicon or photoresist master form stable patterns useful for μCP. Specifically, voids in the stamp of low aspect ratio (the height of the features divided by their lateral dimension <0.2) are susceptible to sagging of the base plane, whereas features of high aspect ratio (>2) exhibit lateral instability (132), (133).

In conventional μCP, a normal pressure is applied to initiate the conformal contact of the stamp over the desired surface. The normal pressure acting on the stamp is concentrated locally on the pattern and posts or lines of the pattern mediate the pressure into the base plane, causing local indentations (134). However, if the pressure applied during μCP is too high the relief features in the stamp can collapse, i.e., the base plane of the stamp comes into contact with the substrate, causing contact in undesired areas. Bietsch and co-workers (134) have demonstrated that model calculations can predict the degree of deformation and the critical pressure of the onset of collapse. Figure 5.1 (a)
shows the modelled deformation for a planar array of square posts 1 \( \mu \text{m} \) wide and 0.45 \( \mu \text{m} \) high, spaced by 4 \( \mu \text{m} \). Compression of the stamp reduces the air gap between the base plane and the printing substrate (Figure 5.1 (b)). When the air gap reaches zero, collapse occurs, in this example predicted at a pressure of 58 kPa (Figure 5.1 (c)).

Figure 5.1 Modelled deformation of a PDMS stamp. (a) Pressure induced deformation of high aspect ratio posts in a PDMS stamp; (b) Front section of (a); (c) Air gap between base plane and surface as a function of applied pressure. Reprinted with permission from (134), Copyright 2000, American Institute of Physics.

The onset of the collapse can be visualized with an optical microscope and results in the formation of an air ring around each post (Figure 5.2).
Increasing the array density (i.e., reducing the spacing of the posts, e.g., from 4 to 2 µm) resulted in stamps more stable, which did not collapse. In the same work, the model was applied to arrays of smaller posts with the same fill factor (area fraction of the printed pattern) which were found to be more stable against normal pressure but also more susceptible to spreading collapse. The authors concluded that, when using stamps with posts with high aspect ratio, large recessed areas have to be avoided and collapse barriers or frames around critical pattern regions should be used to prevent the onset and collapse propagation.

Although stamp collapsing is a disadvantage in most µCP applications, in this Thesis this study exploited rather than avoided it. Therefore, a new stamping method is introduced, denominated ‘microcontact printing trapping air’ (µCPTA), which is based on the use of a stamp having posts of high aspect ratio organized in an array format. This Chapter will present the results for this new micropatterning method applied to patterning micro-arrays of immunoglobulins. The distance between the posts within the array proves to be critical for the outcome of this stamping technique and the potential application to protein microarray technology is discussed. Some early results of this micropatterning technique were presented at a SPIE Conference in December 2005 (see List of Publications).
5.2 MATERIALS AND METHODS

5.2.1 Stamp fabrication

The PDMS stamp was fabricated using PDMS (Sylgard 184, Dow Corning) by replicating a silicon (Si 100) master using the replica molding procedure described in Chapter 3 (Section 3.1.2). The fabrication of the silicon master was outsourced (Institute of Microtechnology, Bucharest, Romania); the desired pattern was generated by photolithography and anisotropic etching (along crystallographic face). The details of pattern on the master will be discussed later in this Chapter.

5.2.2 Master and stamp imaging

5.2.2.1 AFM

Atomic force microscopy (AFM) images were obtained using a Topometrix Explorer (ThermoMicroscopes, Inc.) in non-contact mode. Silicon cantilevers with a spring constant of 42 N/m and resonant frequency of 320 KHz were used in the non-contact mode. Images were obtained using a 100 x 100 µm scanner and the analyses were carried out under air-ambient conditions (temperature of 23 °C and 45% relative humidity).

5.2.2.2 SEM

SEM analysis of the silicon master and the PDMS stamp was performed on a JEOL JSM840 microscope, at 15keV. PDMS stamps were gold-coated prior imaging using a SEM-coating unit E5100 (Polaron Equipment Ltd.) at 25 mA for 90 s at 0.1 Torr.

5.2.2.3 Optical and DIC microscopy

PDMS stamps collapsed over a glass surface were imaged with optical microscopy at the magnification of 20X and then using DIC microscopy (40X) to better resolve the interface between the PDMS and the glass surface. Images were captured using a Coolview FDI high-resolution camera (Photonics Science Ltd.) controlled by Gel-Pro® Plus software (Media Cybernetics).
5.2.3 Protein stamping

Anti-rabbit IgG FITC-labelled was used at the concentration of 50 µg/mL, and adsorbed over the PDMS stamp for 30 minutes. The stamp was subsequently rinsed throughout with PBS 10 mM (3x 100 mL) and MilliQ water (3x 100 mL), dried in air and immediately stamped over a cleaned glass coverslip. Conformal contact was allowed to occur spontaneously and stamping was carried out for 60 seconds, at room temperature, without the use of additional pressure. Stamping was performed over three surfaces, namely glass (24 mm x 24 mm type I glass coverslip), polystyrene (cut out from a Petri dish) and a poly-lysine coated glass slide, the latter kindly provided by CSIRO Molecular Science (Melbourne). The contact angles of these surfaces were measured as an indication of surface hydrophobicity. Contact angles were measured on sessile drops (2 µl) of Nanopure water at room temperature (23 ºC) in air using a Ramé-Hart contact angle goniometer (Model # 100-00-115). Observed values were averaged over six different readings.

In biorecognition experiments, unlabelled rabbit IgG (50 µg/mL) was first stamped over a glass slide, and then reacted for 30 minutes with anti-rabbit IgG FITC labelled (1 µg/mL) or anti-mouse IgG Alexa Fluor 546 labelled (1 µg/mL). The experiment was performed with and without blocking the glass surface with BSA after the stamping step, using a 1 % w/v BSA solution. When blocking was performed, the slide was immersed in the BSA solution for 30 minutes, then rinsed well with buffer and water, and then covered with a solution of anti-rabbit IgG FITC labelled.

5.2.4 Robotic spotting

Robotic spotting was performed at CSIRO Molecular Science (Melbourne) employing a Piezoarray® (Perkin Elmer). In this experiment, the protein solutions (rabbit IgG and anti-rabbit IgG FITC labelled) that were employed were the same as those used for the µCPTA biorecognition experiments. An acrylamide-coated slide (Perkin Elmer) was used during spotting, kindly provided by the host laboratory.
5.2.5 Protein pattern imaging and analysis

5.2.5.1 Laser scanning confocal microscopy (LSCM)

Stamped or robotically-printed fluorescent protein patterns were imaged using the LSCM using the 488 nm or 543 nm laser lines (6% power). For FITC labelled proteins, Channel 1 was used (Ch1: Excitation 488/Emission 500-530); for Alexa Fluor 546 proteins, Channel 2 was used (Ch2: Ex 543/ Em 550-655). For a given dye, the same acquisition settings were used for imaging the patterns, which were captured using the 40X objective.

5.2.5.2 Image analysis

Intensity profiles and intensity surface plots were generated using the LSCM software (FV10-ASW Version 1.3 Olympus) or Image Pro® Plus (Version 5.0, MediaCybernetics®), respectively, using the original confocal images, without any modification. Image Pro® Plus was also used to measure the master, the stamp and the protein pattern dimensions, using a picture of a ruler (captured with the corresponding magnification) for calibrating the image. For any given measure, 10 values were collected and averaged.

Surface contour plots were produced using Surfer Version 8.0 (Golden Software, Inc.) by loading into the software the original confocal images, expressed as $X_i$, $Y_i$, $Z_i$, where $X_i$, $Y_i$ are the Cartesian coordinates of an $i$-pixel and $Z_i$ its intensity. For each processed image, an average background was determined by averaging the signals in the area outside the fluorescent pattern. This value was used as a background threshold ($Z_T$). Finally, for a given pattern, contour plots were generated as signal-to-noise, i.e., as $Z_i/Z_T$.

5.2.5.3 Statistic analysis

The statistical analysis of the fluorescent micropatterns was done as follows. For each image, all the pixels that had a signal intensity ($Z_i$) value lower than the background threshold ($Z_T$) were eliminated. Average ($Z_{\text{average}}$) and standard deviation (SD) of the signal intensity of the remaining pixels were then calculated. The average was used to
obtain the signal to noise ratio \( (Z_{\text{average}}/Z_T) \), whereas the standard deviation was used to describe the homogeneity of the signal.

5.3 RESULTS AND DISCUSSION

The fabrication of the PDMS stamp is illustrated in Figure 5.3, and comprises molding a liquid PDMS pre-polymer against a silicon master lithographically fabricated.

![Fabrication of the PDMS mold](image)

**Figure 5.3** Fabrication of the PDMS mold

The silicon master used for PDMS molding is formed by three series of arrays arranged in three rows, R1, R2 and R3 (Figure 5.4 (a)). Each row includes five arrays of etched V-shaped wells arranged in a 5x5 matrix. The wells forming the arrays in each row have same size but increasing inter-well distance as summarised in Figure 5.4 (b).
Figure 5.4 Details of the silicon master used for PDMS molding. (a) Original sketch of the master; (b) dimensions and inter-well distance of the V-shaped wells in the master.

The dimensions of the etched wells as estimated by AFM are summarised in Table 5.1. AFM and SEM imaging of the three sets of arrays are presented in Figure 5.5 and Figure 5.6, respectively.

<table>
<thead>
<tr>
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<th>Width (µm)</th>
<th>Height (µm)</th>
<th>Aspect Ratio</th>
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<tbody>
<tr>
<td>R1</td>
<td>4.0</td>
<td>3.0</td>
<td>1.33</td>
</tr>
<tr>
<td>R1</td>
<td>5.5</td>
<td>3.5</td>
<td>1.57</td>
</tr>
<tr>
<td>R3</td>
<td>8.5</td>
<td>5.5</td>
<td>1.54</td>
</tr>
</tbody>
</table>

Table 5-1 Dimensions of the V-shaped wells in the silicon master as measured with the AFM.
Figure 5.5 AFM imaging of an array of V-shaped wells in the silicon master used for PDMS molding
**Figure 5.6** SEM images of the silicon master used for PDMS molding. (a) Imaging of single wells; (b) SEM image of an entire array.
5.3.1 Stamp characterization

Replication of the master with PDMS results in a stamp with pyramid-like posts arranged in an array format (Figure 5.7 (A)). By tilting the sample during SEM imaging (Figure 5.7 (B)) the three-dimensional nature of the PDMS pattern can be appreciated, and imaging at high magnification reveals the pyramid-like shape of the posts forming the array (Figure 5.7 (C)). During prolonged SEM imaging, shrinking of the pattern occurs, as clearly detectable in Figure 5.7 ((B) and (C)), an effect that is caused by the high intensity (15 keV) of the electron beam interacting with the PDMS material, and which can be prevented by coating the sample with a thicker gold layer.

Figure 5.7 SEM of PDMS mold. (A) SEM image of an entire PDMS array; (B) the same array imaged tilted by 30°; (C) detail of the pyramid-shaped PDMS posts.
AFM imaging of a PDMS array formed by pyramid-like PDMS post (Figure 5.8) indicates that the replication occurs with excellent fidelity, with less than 10% variation between the dimensions of the master and those of the replica. This loss is due to shrinking of the PDMS mold during the curing step.

Figure 5.8 AFM of a PDMS stamp. (a) Tapping mode micrograph and (b) corresponding line analysis.
The dimensions of the posts in the PDMS stamp obtained after replication as estimated by AFM are summarised in Table 5.2. Comparison of Table 5.1 and Table 5.2 show that the V-shaped wells in the master and the corresponding pyramid-shaped posts in the stamp have practically same aspect ratio, indicating that the shrinking phenomena occurs isotropically.

<table>
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<th>Width (µm)</th>
<th>Height (µm)</th>
<th>Aspect Ratio</th>
</tr>
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<tbody>
<tr>
<td>R1</td>
<td>3.6</td>
<td>2.7</td>
<td>1.33</td>
</tr>
<tr>
<td>R1</td>
<td>5.0</td>
<td>3.1</td>
<td>1.61</td>
</tr>
<tr>
<td>R3</td>
<td>7.8</td>
<td>4.9</td>
<td>1.61</td>
</tr>
</tbody>
</table>

Table 5-2 Dimensions of the pyramid-shaped posts in the PDMS stamp as measured with the AFM.

5.3.1.1 Stamp collapsing and air trapping

When placed in conformal contact with a flat surface (e.g., glass), the pyramid-like posts in the stamp readily collapse, which results in the formation of an air gap between the posts and the receiving substrate. The dimension of the posts as well as their distance within an array dictates how the air gap is formed, i.e., if around the whole array or around each post. Figure 5.9 illustrates this effect. In arrays belonging to ‘R1’ (formed of 3.6 µm posts, Figure 5.9 (a)), an air gap is formed around the entire first array (ARRAY 1.1) but increasing the distance between the posts (ARRAY 1.2) results in the air gap forming around each post, not around the whole array. The situation is different for arrays belonging to ‘R3’ (formed of 7.8 µm posts, Figure 5.9 (b)), which differs from ‘R1’ for the sizes of the posts and their relative distance (as summarised in Table 5.2). Here, the air gap is formed around the first two arrays (ARRAY 3.1 and ARRAY 3.2). Further increasing the distance between the posts results in the formation of the air gap, again, around the single posts (ARRAY 3.3).
Figure 5.9 Transmission light microscope images (20X) showing the collapsing of a PDMS stamp on a flat glass surface.

(a) Collapsing of 3.6 µm posts, in arrays with an inter-well distance of 8, 16 and 32 µm (ARRAY 1.1, ARRAY 1.2 and ARRAY 1.3, respectively); (b) Collapsing of 7.8 µm posts, in arrays with an inter-well distance of 10, 20 and 40 µm (ARRAY 3.1, ARRAY 3.2 and ARRAY 3.3, respectively). Scale bar (a) is 10 µm and in (b) is 15 µm. The two sets of images have different scale bars because they were resized differently.
DIC microscopy allows visualising the interface between the PDMS post and the glass substrate after collapsing (Figure 5.10). When a single post collapses and an air gap is formed around it, the PDMS surface in contact with the glass assumes a star-like shape. This occurs independently on the post size (Figure 5.10 (a)). When the posts are highly packed, a large air gap is formed around the entire array, but the single posts collapsing on the glass surface are distorted in the same way, i.e., forming a star-shaped pattern (Figure 5.10 (b)). As a consequence, stamping with this type of PDMS mold is expected to produce arrays where the single elements have a star-like shape.
Figure 5.10 DIC images of the PDMS stamp collapsed over a flat glass surface. (a) Single features collapsed, with decreased size (left to right); (b) a series of arrays collapsed with an air gap formed around the entire array. Scale bar (in b, right) applies to all images and is 10 µm.
5.3.2 Microcontact printing proteins trapping air

Figure 5.11 and 5.12 show the printing results over a glass substrate from a stamp inked with a solution of FITC-labelled anti-rabbit IgG.

(a)

(b)

**Figure 5.11** μCPTA protein stamping results (I). (a) LSCM image (40X) of a single protein feature stamped (anti-rabbit IgG FITC labelled); (a, inset) LSCM image of the PDMS after stamping; (b) Fluorescence intensity profile corresponding to the dotted line crossing the stamped feature in (a). Scale bar is 10 µm.
Figure 5.12 µCPTA protein stamping results (II). (a) Microarrays of anti-rabbit IgG FITC-labelled printed on a glass substrate. The three arrays were obtained with PDMS arrays having posts about 8, 5 and 3.5 µm wide (A, B and C, respectively). Scale bar in (a, A) is 10 µm and applies to all the images; (b) surface intensity plots corresponding to the arrays shown in (a).
When the posts in the stamp are widely spaced, as expected, collapse of the base plane occurs and the result is a single feature printed on the substrate, together with the area corresponding to the stamping of the base (Figure 5.11). Transfer of the protein layer from the PDMS stamp to the glass substrate is quantitative, as demonstrated from the confocal image of the PDMS post after stamping (Figure 5.11, (a, inset)). The printed feature shows the predicted ‘star-like’ shape, which arises from the deformation of the soft post during stamping. The associated intensity profile in Figure 5.11 (b) shows a drop in the fluorescence emission at the centre of the feature, which corresponds to the tip of the pyramid-like post. This is probably an effect related to the pressure that is exerted at the tip of the post during printing, which might cause a distortion of the tip, and, consequently, less contact with the surface, resulting in less transfer of proteins.

When densely packed arrays are stamped, the base plane collapses around the entire array forming an air gap around it, which prevents printing the areas between the posts. The result is the accurate printing of a protein array with total lack of transfer in unwanted areas (Figure 5.12). For a given post size, the dimension of the correspondent printed area depends on the inter-post distance in the array. Figure 5.13 exemplifies this effect, where ARRAY A and ARRAY B are two sets of arrays belonging to the same row, therefore stamped by posts having same size.
Figure 5.13 Fluorescent confocal image showing two arrays (A and B) of labelled anti-rabbit IgG that were printed using a stamp having same post size (3.5 µm wide) but different inter-post distance (8 µm and 16 µm for array A and B respectively). Scale bar is 10 µm.

Qualitatively, it appears that the printed features in ARRAY A are smaller then those in ARRAY B. The effect observed in Figure 5.13 was consistently observed for all the three rows of arrays (R1, R2 and R3), and appears independent on the size of the posts. A precise measurement of the patterns in Figure 5.13 with graphic software confirms this observation: the features forming ARRAY A are, on average, 3.4±0.3 µm wide whereas the features forming ARRAY B are about 4.4±0.2 µm wide. This effect probably depends on the different pressure that is exerted over the posts forming the two sets of arrays when the stamp collapses. When the posts are widely spaced (ARRAY B), the base plane collapses around the post, which is therefore subject to an isotropic pressure, whereas
when the posts are densely packed (ARRAY A), the base plane collapses around the entire array, therefore the post along the edges of the array are subjected to a higher pressure then the ones in the middle of the array. This effect is clearly visible in the DIC confocal images presented in Figure 5.10 (b) and in the following LSCM fluorescent image (Figure 5.14 (a)), where the printed features at the edges (indicated with an arrow) are visible larger then the central ones.
The intensity profiles across various rows of the array (indicated with 1, 2, 3 and 4 in Figure 5.14 (a)), though, are similar along the three rows, therefore the difference between each pattern is only in their relative size, not in the amount of protein deposited.
5.3.2.1 Influence of substrate type on stamping results

Proteins were printed with µCPTA over different substrates, namely glass, poly-L-lysine and polystyrene. The surface characteristics of these substrates are summarised in Table 5.3.

<table>
<thead>
<tr>
<th></th>
<th>Contact angle (°)</th>
<th>Surface Charge</th>
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<tr>
<td>Glass</td>
<td>38±2</td>
<td>uncharged</td>
</tr>
<tr>
<td>Poly-L-lysine coated glass</td>
<td>55±5</td>
<td>positive</td>
</tr>
<tr>
<td>Polystyrene</td>
<td>83±4</td>
<td>uncharged</td>
</tr>
</tbody>
</table>

**Table 5-3** Surface characteristics of the substrates used in the µCPTA experiments

Among the substrates tested, glass is the most hydrophilic and polystyrene the most hydrophobic, the latter being a polymer often used in protein adsorption applications such as ELISA tests. Glass slides coated with poly-L-lysine are moderately hydrophobic and polycationic, as lysine is a protonated amino acid. The use of poly-L-lysine coated surfaces is an established methodology for DNA microarray technology but their use has also been extended to protein microarray patterning, since the polycationic nature of the substrate allows immobilizing proteins via electrostatic interactions (22), (135).

To compare stamping efficiency over the different substrates, the same protein solution was incubated over three stamps fabricated with the same procedure, and printing over the different substrates simultaneously, under same ambient conditions. As shown in Figure 5.15, protein transfer occurred very well when using plain, hydrophilic glass (Figure 5.15 (A)), less well with poly-lysine (Figure 5.15 (B)) and printing failed when using a hydrophobic surface like polystyrene (Figure 5.15 (C)).
These results are well in accordance with the previously mentioned work of Tan and co-workers (84), who reported obtaining the best printing results for labelled anti-goat IgG when using hydrophilic substrates (e.g., COOH-terminated SAMs), rather than hydrophobic ones (e.g., methyl-terminated SAMs). In Chapter 2 it was already pointed out how this result is counterintuitive, since, in general terms, protein adsorption from solution is higher over hydrophobic surfaces. This suggests a different mechanism involved in the transfer of proteins from the PDMS stamp to the receiving substrate, which is still under investigation (83).

Although other substrates were not investigated, it is likely that surfaces that have been reported as particularly successful in μCP proteins would be also suitable for μCPTA. These include COOH-terminated SAMs, EG₉-terminated SAMs and other hydrophilic surfaces such as amino-terminated glass slides. Alternatively, if printing over a hydrophobic substrate is required, the PDMS mold should be treated to make its surface hydrophilic (e.g., with oxygen plasma).
5.3.2.2 Bioactivity of stamped proteins

Retention of protein bioactivity was investigated through secondary antibody recognition. Printing was performed on plain glass, being this substrate the one that gave the better printing results among those tested.

A freshly prepared PDMS stamp was incubated with a buffered solution of Rabbit IgG (unlabelled) and after rinsing and air-drying, stamped over a clean glass cover slip. After patterning, the substrate was processed for secondary antibody recognition, by exposing it to solution of a labelled anti-rabbit IgG. As a control, a second stamp was coated with Rabbit IgG (unlabelled), rinsed, dried and stamped over a glass slide, and then reacted with anti-mouse IgG Alexa Fluor 546 labelled. Figure 5.16 shows the results for this experiment. Recognition of the stamped rabbit IgG occurs only when the pattern is reacted with anti-rabbit IgG FITC-labelled; when using anti-mouse IgG Alexa Fluor 546 labelled, the stamped patterns remain un-fluorescent, indicating that biorecognition had not occurred, as expected.

In both samples, during the recognition experiment, the labelled IgGs un-specifically adsorbed over the uncoated glass areas. This explains the ‘negative-tone’ fluorescent
image in Figure 5.16 (b). One way to avoid this from happening is to block the un-patterned glass areas against protein adsorption after the \(\mu\)CPTA stamping step, for instance using BSA. This is expected to improve the signal-to-noise of the resulting fluorescent micropattern. The recognition experiment was therefore repeated as follows: a rabbit IgG-coated PDMS stamp was first \(\mu\)CPTA printed, the micropattern was then incubated with BSA (1% w/v) to block unpatterned glass areas, and subsequently it was reacted with the solution of secondary antibody (anti-rabbit IgG FITC labelled). Figure 5.17 shows the results obtained for the recognition experiments performed under two different conditions, i.e. without (a) and with (b) BSA blocking.

![Figure 5.17](image.png)

**Figure 5.17** LSCM images showing secondary antibody recognition results without and with BSA blocking. A printed array of (un-labelled) rabbit IgG was processed with the secondary antibody without BSA-intermediate blocking (a) and with BSA blocking (b). Scale bar in (b, bottom) applies to all images and is 10 \(\mu\)m. Images were acquired using same confocal settings.
The recognition occurs in both cases, indicating bioactivity retention of the printed protein (rabbit IgG). Figure 5.18 shows the intensity profiles associated with the arrays in Figure 5.17 (third row from the bottom of top arrays), where (a) refers to the array processed without BSA blocking and (b) refers to the array processed with BSA blocking.

Figure 5.18 Intensity profiles across the central row of the arrays presented in Figure 5.17 (top), processed without BSA blocking (a) and with BSA blocking (b).

In the first case (a), some unspecific adsorption over the unpatterned glass areas occurs, leading to some background noise (signal-to-noise ratio (S/N) =1.6, calculated as the maximum signal in Figure 5.18 divided by the background noise). Blocking with BSA (b), as expected, prevents non-specific adsorption over the unpatterned glass area,
therefore reducing the background noise with a consequent improvement of the signal-to-noise ratio (S/N=3.1). Another important difference emerges when analysing the 3D surface intensity plots corresponding to the individual features patterned (Figure 5.19).

![Figure 5.19 LSCM images and corresponding surface intensity plots of single patterns of rabbit IgG recognised by anti-rabbit IgG FITC-labelled. (a) Recognition without BSA; (b) recognition with BSA blocking.](image-url)
The BSA-blocked patterns systematically have a dark area in their middle, which extends towards the bottom of the feature, forming an ‘open ring’ shape, clearly visible in Figure 5.19 (b). It was previously noted that, when printing with the µCPTA method using pyramid-like post, less material is transferred in the central area of the feature (see Figure 5.11). Also, blocking the patterned slide with BSA requires immersing the sample in an aqueous solution for 30 minutes, therefore some desorption of the patterned un-labelled protein from the glass slide is likely to occur. These two effects probably account for the resulting asymmetric shape of the patterned features when the BSA blocking method is employed.

To maximize the benefits of the two recognition methods, i.e., pattern accuracy (non-BSA blocking method) and high signal-to-noise ratio (BSA-blocking method), stamping should be performed over a hydrophilic surface where the protein is linked via a stable, covalent bound, such as a COOH-terminated surface. Immobilizing the protein via covalent binding would avoid protein desorption during the blocking step.

5.3.3 µCPTA in protein microarray technology

Robotic spotting is one of the methods currently employed for the fabrication of protein microarrays. As discussed in Chapter 1, the method suffers for its tendency of generating proteins patterns with uneven concentration throughout the spot, creating spots having a typical ‘donut shape’. This effect is apparent in Figure 5.20, which represents a spot of unlabelled rabbit IgG robotically printed and recognized by the secondary antibody, FITC labelled anti-rabbit IgG. The LSCM image was acquired using the same settings used in Figure 5.17. Rabbit IgG was firstly printed over a gel-coated slide, incubated for few hours (to ensure adsorption inside the gel), and then processes for antibody secondary recognition. The concentration, buffer and pH of the primary and secondary antibodies solutions were the same as the ones used in the previously discussed µCPTA experiments, as well as incubation time, and processing conditions.
Figure 5.20 LSCM image of Rabbit IgG mechanically spotted recognised by anti-rabbit IgG FITC-labelled. Confocal image acquired using the same settings used for the contact-printed arrays in Figure 5.16. Scale bar is 40 µm.

Gel-coated slides were chosen as the substrate for robotic spotting since it was recently reported that this type of substrates, having a three-dimensional gel, greatly increase the capacity for the immobilization of proteins compared to flat surfaces (135). Gel-coated slides are therefore considered excellent substrates for antibody microarrays. When spotting over this type of substrates, proteins are retained via unspecific hydrophobic interactions. When performing biomolecule recognition experiments is therefore necessary to block the slide with BSA after protein spotting to prevent unspecific protein adsorption form solution.

5.3.3.1 Comparison between µCPTA and robotic spotting

The comparison between protein patterns generated with the µCPTA method and the robotic spotting method can only be speculative, since the types of substrates used during these experiments were dramatically different, i.e., a glass slide and a hydrogel-coated slide, respectively. The impossibility of using the same type of substrate in these experiments arises from the intrinsic difference of the two patterning method, µCPTA being a stamping technique, where a dry protein monolayer is transferred from the
PDMS stamp to a surface, and the robotic method, which is a spotting technique, where a small volume of protein solution is delivered to a substrate. In the first case, immobilization occurs by physical contact, whereas in the second case proteins physio-adsorb from solution to the substrate. It was already discussed how these two mechanisms appear to be very different, the one involved in µCP yet to be elucidated. Therefore, it would be unrealistic to compare the two technologies using the same type of substrate. It was thus chosen to employ, in each case, a substrate over which patterning gave best results (among the tested substrates), i.e., glass for µCPTA and a hydrogel-coated slide for robotic spotting.

Table 5.4 summarizes the intensity integral, intensity average, and standard deviation for the pattern robotically spotted (Figure 5.20) and for the pattern created with the µCPTA method, with and without BSA blocking (Figure 5.17, (a) and (b) respectively).

<table>
<thead>
<tr>
<th></th>
<th>Intensity (integral)</th>
<th>Intensity average ((Z_{\text{average}}))</th>
<th>Background average ((Z_T))</th>
<th>SD (%)</th>
<th>S/N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spot robotically printed</td>
<td>19775455</td>
<td>366</td>
<td>270</td>
<td>40</td>
<td>1.36</td>
</tr>
<tr>
<td>µCPTA array</td>
<td>1857240</td>
<td>724</td>
<td>570</td>
<td>16</td>
<td>1.31</td>
</tr>
<tr>
<td>(not BSA blocked)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>µCPTA array</td>
<td>1752307</td>
<td>495</td>
<td>250</td>
<td>28</td>
<td>2.2</td>
</tr>
<tr>
<td>(BSA blocked)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 5-4 Statistics of the robotically printed pattern and µCPTA arrays

The diameter of the spot mechanically printed is 185 μm, with a corresponding area of about \(22 \times 10^3\) μm\(^2\), whereas the array printed via µCPTA is 50 μm wide, with an area of one order of magnitude smaller \(2.5 \times 10^3\) μm\(^2\). The single features forming the µCPTA array are about 5 μm wide. As shown in Table 5.5, the intensity integral is higher for the spot robotically printed, which is expected as the respective area is significantly larger then the area obtained by the contact-printed arrays. However, the average intensity signal in the contact-printed arrays is considerably higher then the average calculated for the robotically printed spot. Also, the signal in the contact-printed arrays has lower
variability, as indicated by the corresponding standard deviation percentages. When processing the contact-printed array without blocking with BSA, some unspecific adsorption occurs, which is reflected in a high background noise (about 570 a.u.), which leads to a S/N value of about 1.3 (where S/N here is intended as the average signal within the entire array divided by the average of the background signal). A similar S/N value is obtained for the area that was robotically-spotted. Blocking with BSA, on the other hand, considerably reduces the background noise (down to about 250 a.u.). The result is a higher S/N value (2.2), but the partial desorption of the contact-stamped proteins during the BSA blocking step leads to a more variable signal, with a standard deviation percentage of 28%. This value is still considerably lower than the standard deviation percentage of the robotically spotted pattern (40%).

Figure 5.21 shows the surface contour plot corresponding to the LSCM image in Figure 5.20, which is the mechanically spotted unlabeled rabbit IgG recognized by the FITC-labelled anti-rabbit IgG. For comparison, the surface contour plots of the µCPTA arrays in Figure 5.17, without BSA blocking and with BSA blocking, are also shown. The surface contour plots represent the variation of the signal-to-noise within the pattern area. For the spot robotically printed (Figure 5.21 (a)), S/N varies considerably throughout the spotted area, but is definitively lower in the middle area and higher along the spot edges, leading to the typical ‘donut shape’ of the signal. In contrast, the surface plots corresponding to the arrays that were created with the µCPTA method show very little variation of the signal-to-noise ratio throughout the arrays pattern. The asymmetry of the patterns in Figure 5.21 (c), as previously discussed, is the consequence of partial desorbing of the patterned protein during the BSA block step (see also Figure 5.19).

The homogeneity of the signal throughout the stamped arrays can also be appreciated in Figure 5.22, which shows the little variation of S/N ((a) and (b)) and of the standard deviation percentage ((c) and (d)) among the single 25 patterns composing the non-BSA blocked ((a) and (c)) and BSA-blocked ((b) and (d)) arrays.

Therefore, while the robotically printed pattern presents a highly variable signal throughout its area, concentrated along the spot edges, with a consequent high standard deviation percentage, the arrays contact-printed are formed by a series of patterns (25 in
this specific example) with similar shape, having a higher signal, which is also significantly more uniform.

Figure 5.21 Surface contour plot of signal-to-noise (S/N) corresponding to (a) the protein spot robotically printed (b) non-BSA blocked μCPTA printed and (c) BSA-blocked array μCPTA. Images are not to scale.
Non-BSA blocked

BSA blocked

(continued on next page)
Figure 5.22 Signal-to-noise ((a) and (b)) and standard deviation percentage ((c) and (d)) across each pattern forming the, non-BSA blocked ((a) and (c)) and BSA blocked ((b) and (d)) protein array. The red dotted line in each plot shows the corresponding average result for the pattern robotically spotted. Colours in the plots do not correspond to different set of data but are only used for visualization.
5.3.3.2 \( \mu \text{CPTA} \) applied to microarray technology

The possible transition from robotic spotting to \( \mu \text{CPTA} \) printing is schematised in Figure 5.23, where a single spot robotically printed is replaced by a contact-printed spot having a series of micro-patterns organised in an array, and enclosed in a ring. By doing so, a single spot robotically printed (typically around 150 \( \mu \text{m} \) wide) can be broken up in a true micro-array (which can be considerably smaller than a single robotically printed spot, e.g., 50 \( \mu \text{m} \) wide or smaller), formed by an arbitrary number of micro-patterns, each being few microns wide. To produce such a contact-printed pattern a two-level PDMS stamp is required, which can be easily fabricated with established multi-level soft-lithographic methods (136).

![Figure 5.23](image)

**Figure 5.23** Schematic representation illustrating the transition from a robotically spotted protein microarray to the \( \mu \text{CPTA} \) technique.

In principle, classic \( \mu \text{CP} \) would give a similar result (i.e., a printed microarray) but \( \mu \text{CPTA} \) allows the formation of a microarray enclosed in a ring (indicated as ‘A’ in Figure 5.23), which can be easily visualized with a conventional microarray reader even
at low (20X) magnification. Rather then reading the intensity signal of the whole spot as
in conventional microarray robotic technology, here a region of interest can be selected
and the reading done including only the printed microarray (the dashed area ‘B’ in
Figure 5.23). Therefore, the ring around the array serves only as a guide to find the
micro-array and is not included in the reading. This eliminates an area that is likely to
have a non-uniform signal due to uneven printing of the stamp along its edges. The
region of interest within the spot, being formed of a micro-array generated by printing a
lithographically made stamp, is spatially addressable, i.e., the geometry of the array is
known and each pattern composing the array can be individually addressed.

Finally, in conventional microarray technology the high variability of the signal within a
printed spot, and the numerous variables affecting the printing outcome, result in the
need for printing repetitions of a same sample within an array. This problem can also be
easily solved using a µCPTA-stamped array, being this formed of numerous patterns
uniform in shape and signal, which can be read individually and treated as repetitions.

5.3.4 Conclusions

A new microcontact stamping method was developed and its application to protein
patterning was demonstrated. Compared with robotic spotting, where single spots of
about 150 μm are created, the method allows to pattern proteins in the form of a spot
(about 50 μm in diameter or less) having, in its interior, an array of micro-patterns. This
way, an ‘array of arrays’ can be easily fabricated. The signal throughout this microarray
has a higher S/N and is less variable compared to the signal in the mechanically-
generated spot. Also, each spot generated with µCPTA is formed of an array of
elements which have similar S/N and standard deviation, and which can be used as
independent repetitions. This eliminates the need to have repetitions during printing,
thus reducing the overall space of the patterned surface and reducing the amount of
sample used.

µCPTA follows the same general rules of µCP, i.e., if an untreated stamp is used, the
best printing results are obtained on hydrophilic surfaces, whereas the contrary is true if
the stamp is oxidized and rendered hydrophilic. In the work reported in this Thesis, the
technique was demonstrated using glass as the receiving substrate. Printed antibodies
remained bioactive, as demonstrated by their ability to recognize selectively their corresponding antigen. Although printing yielded excellent results over the glass substrate, when the stamped array was processes for biorecognition, proteins adsorbed non-specifically to the un-patterned glass surface, with a consequent effect on S/N. Blocking with BSA could prevent this from happening, but some desorption of the stamped proteins occurred. For the method to be most effective, in terms of pattern stability and selectively, proteins should be patterned over a carboxyl- or amino-terminated surface with the use of a cross-linker to produce covalent binding, and then the substrate blocked with BSA before further processing.
6 THE BEADMICROARRAY PLATFORM

6.1 INTRODUCTION

Microbeads can be used as sensing elements for the spatial immobilization of proteins. Numerous works that use this approach for the fabrication of positional microarrays were previously reviewed in Chapter 2 (Section 2.4.4). Microbeads can be arranged in an array format either to form spots of agglomerated beads or as single beads. As previously discussed, single beads are advantageous compared to agglomerated beads, since in the latter case the number of aggregated beads is unknown and, consequently, the surface density available for immobilization is difficult or impossible to predict and control.

Currently, there are two methods for fabricating arrays of single microbeads: the positioning of beads inside an array of micro-wells, or the positioning of the beads at the end of a bundle of microcapillaries, such as the etched end of a bundle of optical fibres. The latter is the technique employed for the fabrication of the BeadArray™, a commercial optical sensor that uses microbeads as sensing elements. In both cases, the ratio between the dimension of the well (or microcapillary) and the dimension of the microbead dictates the number of beads that will be entrapped at each site.

This Chapter reports a new method for fabricating an array of microbeads. The method combines knowledge on the self-assembling of microbeads in microfabricated structures with PDMS replica molding. The resulting platform is denominated ‘BeadMicroArray’. Although this type of array can be used for the immobilization of any biomolecule, its specific application to protein immobilization is discussed. An Australian Provisional Patent (application No. 2006903471, see ‘List of Publications’) has been filed in relation to this novel method for fabricating micro- and nanoarrays.
6.2 MATERIALS AND METHODS

6.2.1 Materials

Glass microspheres (2-10 µm, powder) were purchased from Polysciences, Inc. Amino-terminated melamine microspheres (NH$_2$-microspheres) were kindly provided by Corpuscular, Inc. (Mahopac, NY, USA), as a 2% wt. water suspension. Ethanol (HPLC grade), acetone (HPLC grade) and DMSO (>99.9%) were purchased from Sigma Aldrich.

6.2.2 Protein solutions

BSA-Alexa Fluor 546, streptavidin-Alexa Fluor 488, and anti-mouse IgG-Alexa Fluor 546 were used at the working concentration of 50 µg/ml. PBS 10 mM (pH 7.4) was used as the dilution and washing buffer.

6.2.3 Bead solutions

All bead solutions were prepared at the approximate concentration of 0.04 wt. % using MilliQ water. Solutions were always prepared fresh to minimize the formation of aggregates. Prior to their use, beads were vortexed for 1-2 minutes using a Vortex-Genie 2 (Scientific Industries, Inc.).

6.2.4 Bead biotinylation

Biotin was covalently attached to the amine terminus of NH$_2$-microspheres by reacting them with EZ sulfo-NHS-LC-biotin (Pierce Chemicals). 15 µg of biotin-ligand (MW 556.59) were dissolved in 40 µL of water and the solution immediately added to 60 µL of the stock solution of NH$_2$-microbeads, to a final concentration of 0.3 M. The mixture was shaken well and then left to react for 30 minutes at room temperature under gentle shaking. Beads were then centrifuged (10,000 rpm, 2 min), the biotin-solution removed and the beads washed well with milliQ water. Finally, 200 µL of milliQ water were added to biotin-microspheres, to a final concentration of about 0.3 % wt.

To confirm that biotinylation of the beads had occurred, the beads were reacted with streptavidin Alexa Fluor 488 at the concentration of 50 µg/mL (see below).
6.2.5 Protein adsorption over beads

BSA-Alexa Fluor 546 (0.1 mg/mL) and streptavidin-Alexa Fluor 488 (50 µg/mL) were non-covalently adsorbed over the surface of amino-terminated melamine microspheres by placing a sample of microbeads (5 µL of the stock 2 % wt-solution) in 250 µL of the protein solution. The solution was reacted for 30 minutes, in the dark, at room temperature under rotation. After reaction, the protein solution was separated through centrifugation (10,000 rpm, 2 minutes) and the resulting wet cake washed three times with buffer. At the end of washing, beads were re-suspended in buffer.

6.2.5.1 BSA blocking

To obtain complete blocking, a BSA solution of 1 % w/v (10 mg/mL) in PBS 10 mM was used. Blocking was performed as described above, by adding BSA to a small solution of microbeads, and leaving to react under rotation. The beads were then centrifuged and washed as described above, using PBS as the washing buffer. BSA-blocked beads were then reacted with a second labelled protein to test the blocking efficiency.

6.2.5.2 Biorecognition

Streptavidin Alexa Fluor 488 (150 µL, 50 µg/mL) was added to a sample of biotin-microbeads and reacted at room temperature, in the dark, for 30 minutes while rotating. The protein solution was then centrifuged, the supernatant removed and the wet cake washed well with buffer and water. The streptavidin-biotin-microspheres were then re-suspended in buffer and kept at 4° C.

6.2.6 BeadMicroArray fabrication

6.2.6.1 Master fabrication

The silicon master employed in this work is the same one used in Chapter 5. Single chips of about 1x1 cm were used in each experiment, every chip containing about 60 etched microarrays.
6.2.6.2 Bead deposition

A silicon master (single chip) was sonicated in ethanol for 5 minutes to remove dust, and then dried in oven. Beads were deposited inside the etched wells of the silicon master by spin coating, using a Specialty Coating Systems spin coater (Model P6708). A volume (150 µL) of the bead solution (0.04 % wt) was deposited over the silicon master and the rotor spun to 500 rpm. After 30 seconds, the speed was increased to 2000 rpm and spinning kept for another 60 seconds. Spinning was repeated three times. Between each spin, a thin, flat piece of PDMS was placed over the master, to remove beads that had deposited over the silicon surface.

6.2.6.3 PDMS Replica molding

The silicon master was placed at the bottom of a 12-well polystyrene plate (one chip per well). A piece of aluminium foil was placed at the bottom of the polystyrene well so that the chip could be removed from the plate after molding. PDMS was prepared as previously reported (Chapter 3, Section 3.1.2), i.e., mixing well 10 parts of the pre-polymer with 1 part of curing agent. About 2 mL of pre-polymer solution was used per silicon chip. After degassing for 1 hour under vacuum, the solution was gently poured over the silicon chip and cured at 60° C for two hours to ensure complete cross-linking. The PDMS replica was then gently cut and removed from the master. After replica molding, the master could be removed from the polystyrene plate and re-used.

6.2.7 BeadMicroArray characterization and modification

6.2.7.1 Mechanical stability

The same BeadMicroArray platform was sonicated (separately) in a 20 mL of PBS, ethanol and acetone for 5, 10 and 20 minutes using an ultrasonic cleaner (Unisonics, Pty. Ltd.). Between each wash, the platform was imaged with the 40X objective of an upright microscope and the number and position of the beads entrapped in the PDMS mold (12 in total) recorded.

6.2.7.2 Reactivity

The amino-reactive probe Alexa Fluor 546 was used to test the reactivity of a BeadMicroArray fabricated with NH$_2$-microspheres. The PDMS stamp was covered
with 150 µL of the dye solution (0.4 mg/mL in DMSO) and left reacting for 1 hour. The stamp was then washed with DMSO, water and air dried. A BeadMicroArray fabricated with glass microspheres was used as a control sample and reacted with the dye under the same conditions.

6.2.7.3 Biorecognition

A BeadMicroArray platform fabricated with biotin-terminated beads was first blocked with BSA and then reacted with streptavidin-Alexa Fluor 488. Blocking was performed by covering the stamp with BSA (1 % w/v solution) left reacting for 30 minutes. The BSA solution was then removed and the stamp washed well with PBS (3x100 mL) and MilliQ water (3x100 mL). The stamp was finally air dried and immediately used for biorecognition. A volume (150 µL) of streptavidin Alexa Fluor 488 was (50 µg/mL) placed over the stamp and left to react in the dark, at room temperature for 30 minutes. The PDMS stamp was then washed well with buffer (3x100 mL) and milliQ water (3x100 mL). Prior imaging, the stamp was air dried. An identical platform was BSA-blocked and exposed to a solution of anti-mouse IgG Alexa Fluor 546 labelled and used as a control.

6.2.8 Optical microscopy and laser scanning confocal microscopy

6.2.8.1 Beads

A solution (about 20 µL) of beads was placed at the edge of a flow cell, formed by a glass cover slip (24x24 cm) placed over a clean thin microscope glass slide (24x60 cm) with two parallel strips of double-sided tape placed at 20 mm apart.

Laser scanning confocal microscopy was used to image the beads, using a 40X oil objective. Beads carrying adsorbed proteins were imaged using the 488 nm or 543 nm laser lines. For FITC (or Alexa Fluor 488) labelled proteins, Channel 1 was used (Ch1: Excitation 488/Emission 500-530), when labelled with Alexa Fluor 546, Channel 2 was used (Ch2: Ex 543/ Em 550-655).

When imaging beads that had two adsorbed proteins carrying two different dyes (e.g., Alexa Fluor 488 and Alexa Fluor 546), the two optical channels were used
simultaneously using a suitable dichroic mirror (SDM 560 nm) placed between the two channels. All images were acquired using same gain and voltage; therefore images can be directly compared.

6.2.8.2 BeadMicroArray platform.

The PDMS stamp was placed over a thin microscope glass slide (24x60 cm). To prevent the collapsing of the stamp, the stamp was placed over two parallel strips of double-sided tape placed at a distance slightly smaller the width of the stamp, with its left and right edges placed over the left and right strip, respectively. The height of the double-sided tape ensured that the stamp did not collapse and did not complicate visualization of the stamp at the inverted microscope (with the exception of the 40 X oil objective, as discussed below).

The BeadMicroArray platform (unstained) was visualized with DIC microscopy using a 60 X water objective mounted on the optical inverted microscope and capturing the images using the CCD camera.

Fluorescent images of the BeadMicroArray were captured with the LSCM, using a 20 X dry objective. Higher magnification on this microscope could not be used due to the short working distance of the other objectives (40X and 100X) mounted on the LSCM, which could not focus properly the BeadMicroArray. To obviate to this inconvenience, the 20 X objective was used together with the zoom-in capability of the microscope.

A BeadMicroArray having fluorescent beads was imaged using the same laser power and channel settings described above for the ‘free’ fluorescent beads (Section 6.2.8.1). The acquisition settings (gain and voltage) were changed and optimized to obtain the best possible images. When imaging this type of BeadMicroArray, the focus was placed over the beads.

Confocal microscopy cannot be used together with DIC optics, thus—compared to the images acquired with DIC microscopy— the confocal images appear slightly de-focused, especially when using the zoom-in factor.
6.2.9 Scanning electron microscopy

SEM analysis of the BeadMicroArray was performed on a JEOL microscope (JSM840) at 15keV. Prior to SEM imaging, the PDMS stamps were coated with gold using Gold coating using a SEM-coating unit E5100 (Polaron Equipment Ltd.) at 25 mA for 90 s at 0.1 Torr.

6.3 RESULTS AND DISCUSSION

6.3.1 Fabrication of the BeadMicroArray

The fabrication scheme for the BeadMicroArray is illustrated in Figure 6.1.

A water solution of beads is spin coated over a lithographically fabricated silicon master. The master used in this work is the same previously described in Chapter 5, i.e., a series of 25 V-shaped wells arranged in an array format. The master comprises three rows of arrays, denominated R1, R2 and R3, which differ for the dimension of the V-
shaped wells. Each row contains five arrays, characterized by different inter-well distance. The master dimensions are summarized in Table 6.1.

<table>
<thead>
<tr>
<th>Array Type</th>
<th>Dimension of the V-shaped wells (µm)</th>
<th>Well depth (µm)</th>
<th>Inter-well distance (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>R1</td>
<td>4</td>
<td>3</td>
<td>8/ 16/32/64/128</td>
</tr>
<tr>
<td>R2</td>
<td>5.5</td>
<td>3.5</td>
<td>6/ 12/24/48/96</td>
</tr>
<tr>
<td>R3</td>
<td>8.5</td>
<td>3.5</td>
<td>10/20/40/80/160</td>
</tr>
</tbody>
</table>

Table 6-1 Summary of dimensions of the wells in the silicon master

During spin coating, the beads are randomly distributed in the wells of the master. Spin coating is repeated three times to ensure a good coverage, and the silicon wafer is pressed against a flat piece of PDMS between each coating step. PDMS pre-polymer is then poured over the silicon master, cured and peeled away form the master. The resulting PDMS stamp is identical to the one obtained and described in Chapter 5 (pyramid-like posts), with the difference that beads are entrapped at the tip of the posts. If a second PDMS mold is fabricated using the same silicon master, no beads are found entrapped in the mold; thus, beads are transferred from the silicon master to the PDMS mold quantitatively.

A mixture of glass beads having dimensions ranging from 2 to 10 microns was used to demonstrate the fabrication method and to evaluate how the bead size influences the array outcome. Figure 6.2 (A-C) shows SEM images of a typical array fabricated using this mixture of glass beads.
Figure 6.2 (A-C): SEM images of the BeadMicroArray fabricated using a mixture of glass beads (2-10 µm); the array in image C is the same of the one in image A, tilted 30°; D: schematic representation of the effect of bead dimension on its positioning inside the PDMS mold.

While smaller beads are embedded close to the end of the diamond tip (solid arrow in Figure 6.2 (C), the bigger beads are collapsed inside them (dashed arrow in Figure 6.2 (C)). This is due to the different positioning of the two types of beads, as schematized in Figure 6.2 (D). Based on these results a homogeneous solution of 2 µm beads was chosen for further fabrication.

6.3.1.1 Microscope imaging

The three-dimensional nature of the BeadMicroArray makes DIC microscopy particularly suitable for its imaging. As shown in Figure 6.3, while with normal optical
microscopy the beads at the top of the PDMS posts are barely visible, with DIC microscopy the beads can be well imaged.

**Figure 6.3** Beads entrapped in a PDMS mold imaged optical microscopy (left) and DIC microscopy (right). Scale bar is 5 µm and applies to both images.

When using DIC optics, focusing the sample at different points leads to very different images. As visible in Figure 6.4, both DIC images, the appearance of the beads is very different when focusing on the PDMS surface (Figure 6.4 (a)) or on beads surface (Figure 6.4 (b)). Images that are presented in this Chapter were collected by placing the focus on the microspheres surface.

**Figure 6.4** Effect of focus change on the imaging of a BeadMicroArray using DIC optics. Scale bar is 10 µm and applies to both images.
6.3.1.2  Effect of well size on bead-cluster formation

A PDMS stamp was fabricated as described above using a diluted solution of 2 µm microspheres and the resulting 2µm-BeadMicroArray (2µm-BMA) was imaged with DIC microscopy (Figure 6.5).

Figure 6.5 Bead clusters inside the three types of arrays, R1, R2 and R3. Scale bar (in R3, left) is 10 µm and applies to all images.
In the array with larger posts, corresponding to R3, more than one bead is often encountered per post (Figure 6.5 (R3)). In the array formed by intermediate posts (R2), occasionally, multiple beads are entrapped within the same post, but mostly single beads are encountered (Figure 6.5 (R2)). In the array formed by smallest posts, only a single bead is entrapped per post (Figure 6.5 (R1)). Clearly, the ratio between the well diameter (D) and the microbead diameter (d) dictates the number of beads that are deposited per well (for a given well depth). This result was previously reported by Yin and co-workers (72) and discussed in Chapter 2, where it was shown how these authors controlled D/d to fabricate specific microbead-clusters (Table 6.2; see also Figure 2.7).

The depth of the micro-well also plays a role in determining the geometry of the cluster, i.e., single layered or double layered. In the cited work, a substrate with cylindrical micro-wells was used and double-layered clustered could be formed under specific conditions. In this specific work, using 2 µm beads, double-layered clusters were not formed due to the V-shape geometry of the micro-wells.

<table>
<thead>
<tr>
<th>D/d</th>
<th>Single-layered (0.5d ≤ H ≤ 1.37d)</th>
<th>Double-layered (1.57d ≤ H ≤ 2.23d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.00 - 2.00</td>
<td>microspher</td>
<td>dimer</td>
</tr>
<tr>
<td>2.00 - 2.15</td>
<td>dimmer</td>
<td>tetrahedron</td>
</tr>
<tr>
<td>2.15 - 2.41</td>
<td>triangle</td>
<td>octahedron</td>
</tr>
<tr>
<td>2.41 - 2.70</td>
<td>square</td>
<td>bisquarepyramid</td>
</tr>
<tr>
<td>2.70 - 3.00</td>
<td>pentagon</td>
<td></td>
</tr>
<tr>
<td>3.00 - 3.30</td>
<td>hexagon</td>
<td></td>
</tr>
</tbody>
</table>

Table 6-2 Cluster type depending on the ratio between well and microbead dimensions. (D, H): dimensions of the wells; and (d): diameter of the microbeads. Reprinted with permission from (72) Copyright 2001 Wiley-VCH Verlag GmbH.

The number of beads deposited inside the silicon master during spinning dictates the number of beads embedded in the resulting PDMS mold. Table 6.3 summarizes the
results for a number of sampled PDMS arrays belonging to R1, R2 and R3. For arrays belonging to R1 (a row of arrays having pyramid-like posts about 3.5 µm wide), posts with more than one bead are never encountered. In arrays belonging to R2 (a row of arrays having pyramid-like posts 5 µm wide) more than one bead per post are encountered in less than 10% of the cases; for arrays belonging to R3 (a row of arrays having pyramid-like posts about 8 µm wide), this percentage reaches 100%. The average number of posts with a single bead in arrays belonging to R1 and R2 is the same, i.e., 13, about half the total posts forming the array (25 in total). In arrays belonging to R3, this number is lower, but the total number of occupied posts is higher, due to the presence of numerous posts with more than one bead. The higher number of occupied posts in R3 reflects a greater ‘chance’ of the beads to fall in the larger wells during spinning.

<table>
<thead>
<tr>
<th>Row type</th>
<th>Number of arrays sampled</th>
<th>% of samples with a single post with more than 1 bead</th>
<th>Average number of posts with a single bead (± SD)</th>
<th>Average number of total occupied posts (± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>R1</td>
<td>20</td>
<td>0</td>
<td>13 ± 3</td>
<td>13 ± 3</td>
</tr>
<tr>
<td>R2</td>
<td>15</td>
<td>9</td>
<td>13 ± 3</td>
<td>13 ± 3</td>
</tr>
<tr>
<td>R3</td>
<td>15</td>
<td>100</td>
<td>7 ± 2</td>
<td>17 ± 2</td>
</tr>
</tbody>
</table>

**Table 6-3** Statistics of beads entrapped in arrays belonging to rows R1, R2, R3.
6.3.2 BeadMicroArray encoding

Spin coating was chosen as the deposition method in order to achieve partial and random deposition of the beads in the micro wells. This way, within an array of 25 V-shaped wells, only a portion is filled with the microbeads, the rest of the wells remaining empty.

As discussed above, only single beads are deposited inside the smallest wells (R1), therefore making this size the best suited for fabricating arrays of single beads. The distribution of the beads inside the V-shaped wells is totally random, thus after PDMS replication, each array in the mold is characterized by a specific code, corresponding to the positioning of the beads within the array. Therefore, identical platforms with different codes can be easily fabricated (Figure 6.6).

![Figure 6.6 DIC images showing three different codes in the same type of array. Scale bar is 10 μm and applies to all images.](image-url)
6.3.3 Functional BeadMicroArray

In order to fabricate a BeadMicroArray suitable for protein immobilization, functional beads bearing suitable chemical groups need to be used during the fabrication of the array. Immobilization can be done through spontaneous adsorption or covalent binding, in which case carboxyl- or amino-terminated beads provide suitable anchoring points for protein immobilization. In this work, covalent binding of proteins was sought and amino-terminated beads were chosen.

The beads employed are melamine amino-terminated microspheres, which are characterized by a high and uniform ligand density (according to the manufacturer, 12.6x10^6 amino groups per microsphere). The beads are spherical, uniform in dimension (2 µm), and have a limited tendency to aggregate, as shown in Figure 6.7. The use of diluted solution of beads (0.04 % wt) and gentle vortexing prior their use limits the formation of aggregates.

![Figure 6.7 SEM of aminated melamine microspheres (courtesy of Corpuscular Inc., (Mahopac, NY, USA)).](image-url)
The density of the microspheres is important when using spin coating as the deposition technique. While the flow cell system (a scheme of which was reported in Chapter 2, Section 2.4.4.2) allows depositing low density beads such as polystyrene beads (d=1.04 g/cm$^3$), when spin coating is used, the beads need to have higher density. In the latter case, silica beads (d=2g/cm$^3$) are normally employed (70). Melamine beads (d=1.51 g/cm$^3$) are preferable also because their density is higher than that of polystyrene beads. However, compared with silica beads, melamine beads have lower density, but this did not represent a problem for their deposition via spin coating. In theory, magnetic beads can also be employed; practically, their use is limited by their intrinsic tendency to aggregate.

NH$_2$-microspheres were entrapped in a PDMS mold using the same fabrication process described previously. When using NH$_2$-microspheres, the resulting PDMS array is indicated as NH$_2$-BeadMicroArray.

**6.3.3.1 Mechanical stability**

The NH$_2$-BeadMicroArray was exposed to extensive sonicating to assess the mechanical stability of the trapped beads. Different media were used and sonication was done for 5, 10 and 20 minutes. The media that were chosen are the ones commonly used when performing protein immobilization (aqueous buffers) and when cleaning the substrate (ethanol), including an (unlikely to be used) organic solvent (acetone). Out of the 12 beads forming the array, no bead detached from the PDMS mold, even after 20 minutes of sonicating. Therefore it is safe to say that, once embedded in the PDMS mold, the beads are permanently trapped.
6.3.3.2 Reactivity

In order for the NH$_2$-BeadMicroArray to be suitable for biomolecule immobilization, it is crucial that the fabrication process does not alter the chemical functionality of the beads. Therefore, the reactivity of the NH$_2$-microbeads after the array fabrication was tested using an amino-reactive fluorophore (2); an array of glass beads was used as a control. Figure 6.8 shows the results obtained after reaction of the NH$_2$-BMA with a solution of the amino-reactive dye Alexa Fluor 546.

![Fluorescence images](image)

**Figure 6.8** Epi-fluorescence microscopy images after the reaction of the NH$_2$-BeadMicroArray with Alexa Fluor 546. (a): control, i.e., BeadMicroArray with trapped glass microspheres; (b): NH$_2$-BeadMicroArray.

Fluorescent beads were detected exclusively on the amino-functionalized array (not in the control). In the epi-fluorescence image of the control the glass beads are barely visible. Unfortunately, DIC optics cannot be used together with the fluorescence optical settings.

This result shows that the functionality of amino-terminated beads is left intact during the fabrication process. Although it is reasonable to expect that beads with other functionalities would remain stable as well, this needs to be confirmed case by case.
6.3.3.3 **Attachment of ligands—general considerations**

The NH$_2$-microspheres can be functionalized with a ligand (e.g., biotin), either before or after their immobilization inside the PDMS mold. If beads are modified before fabrication the stability of the ligand during the replication process must be considered since PDMS replica molding involves the use of a pre-polymer which contains organic monomers and solvents, as well as curing at 60° C. However (as mentioned in Chapter 3) PDMS can be cured at temperatures lower then 60° C, and if necessary, curing at room temperature is also possible (with the drawback of longer curing time).

An the other hand, if the beads are first entrapped in the PDMS mold and then functionalized with the desired ligand (e.g., biotin), the interaction of the ligand with the PDMS surface must be evaluated and, if necessary, blocked. It should be noted that blocking is necessary only if the ligand can attach to the PDMS surface. PDMS is chemically inert, i.e., it is not reactive towards chemical groups like amino, carboxyl and thiol groups. Indeed, oxidation is required to chemically modify the surface properties of PDMS and render it reactive. Thus, blocking is not required when working with peptides (e.g., oligonucleotides) or other biomolecules (e.g., carbohydrates). In contrast, it represents an issue if the ‘ligand’ is a protein, since the hydrophobic nature of PDMS favours protein adsorption.

6.3.4 **The BeadMicroArray platform applied to the spatial addressable immobilization of proteins**

The following section presents results for the NH$_2$-BeadMicroArray applied to the spatial, addressable immobilization of proteins. The amino functionalities of the microspheres can be used for covalent binding, e.g., modifying the beads with a cross-linker (e.g., glutaraldehyde), or biorecognition, e.g., attaching a biotin-ligand to the microspheres. In both cases, the PDMS component of the NH$_2$-BeadMicroArray platform needs to be blocked against non-specific protein adsorption.

PDMS can be blocked using BSA. When working with a NH$_2$-BeadMicroArray platform, though, it is necessary to consider also the adsorption of BSA over the second component of the array, i.e., the NH$_2$-microspheres. Ideally, the adsorption should be limited only to the PDMS surface, in order to leave the surface of the NH$_2$-microspheres available for binding.
Amino-terminated surfaces are moderately hydrophilic and generally repel protein adsorption. For instance, when patterning a polymer that generates amino groups upon exposure to UV light, we have found preferential adsorption over the unexposed regions (137).

Although amino-terminated surfaces generally repel protein adsorption, this is possibly not the case with BSA, a protein which is known to adsorb non-specifically to numerous surfaces, including hydrophilic ones. Therefore, BSA interaction with the NH₂-microspheres was studied separately.

### 6.3.4.1.1 BSA adsorption over the NH₂-microspheres

The adsorption of BSA over NH₂-microspheres was studied by adsorbing, sequentially, BSA and streptavidin labelled with two different dyes and using the confocal microscope to image both dyes. Labelled BSA (BSA-Alexa Fluor 546, 0.1 mg/ml) was first adsorbed over a sample of amino-terminated beads; after repeated washing with buffer, the beads were imaged with the confocal microscope. Following the BSA adsorption, the beads were reacted with a solution of labelled streptavidin (streptavidin-Alexa Fluor 488). After incubation, centrifugation and washing with buffer, the beads were imaged again with the confocal microscope. Both samples were imaged using two channels, i.e., Channel 1 and Channel 2, set to detect the two dyes (Ch1: Alexa Fluor 488, Ch2: Alexa Fluor 546). A third control sample of amino-terminated beads was imaged using the two-channel settings; all images were acquired using same settings (Figure 6.9).
Figure 6.9 LSCM images showing results of BSA adsorption over NH$_2$-microspheres. (a): control; (b): microbeads after reaction with BSA-Alexa Fluor 546; and (c) microbeads after subsequent reaction with streptavidin Alexa Fluor 488. TR=transmission light; Ch1= Ex 488 nm/Em 500-530; Ch2= Ex 543 nm/Em550-655. Scale bar in (a, left) is 10 µm and applies to all images.
After reaction with BSA-Alexa Fluor 546, the NH$_2$-microspheres become fluorescent, an indication that the protein had adsorbed over the beads surface. The uncoated beads (control) are non-fluorescent in both channels; therefore the fluorescent signal in Ch2 is univocally assigned to the Alexa Fluor 546 dye. Also, no signal in Ch1 is detected for both samples (i.e., BSA-Alexa Fluor 546-coated spheres and control). Reaction of the BSA-Alexa Fluor 546 coated microbeads with streptavidin-Alexa Fluor 488 leads to beads that are fluorescent in both channels, indicating that both proteins, BSA and streptavidin, are adsorbed over the beads. Based on these results it can be concluded that BSA adsorbs over the NH$_2$-microspheres, but, at this concentration (0.1 mg/ml) the coverage is non-uniform, leaving sites available for the adsorption of a second protein (i.e., streptavidin). By increasing the concentration of BSA (10 mg/ml, i.e., 1% w/v), a higher surface coverage is obtained, which is reflected in an increased blocking efficiency towards the adsorption of the second protein. This is illustrated in Figure 6.10, a confocal image (using Ch1) of a sample of beads reacted first with a high concentration (10 mg/ml) of unlabelled BSA and subsequently (after extensive washing), with streptavidin-Alexa Fluor 488. In the latter case, the higher concentration of BSA leads to a higher coverage of the NH$_2$-microspheres, thus reducing considerably the sites available for the adsorption of streptavidin. The consequence is a lower fluorescent signal in Figure 6.10 (Ch1) compared to the signal in Figure 6.9 (bottom row, Ch1).

---

4 Acquired using same settings as those used in Figure 6.9
In conclusion, BSA adsorbs on the amino-terminated microspheres, but when using a low concentration (0.1 mg/mL), the coverage is only partial, leaving sites on the surface of the beads available for the attachment of a second protein (or another ligand). When using a higher concentration (10 mg/mL), the beads are totally blocked and their surface ‘masked’ by the protein.

6.3.4.1.2 Blocking a NH$_2$-BeadMicroArray platform against non-specific protein adsorption

The previous results can be summarized as follows:

1. BSA can be used to block the non-specific adsorption of proteins over the PDMS surface but the protein also adsorbs to the NH$_2$-microspheres.

2. When blocking is performed using a BSA solution at low concentration (0.1 mg/mL) the coverage of the NH$_2$-microspheres is not complete, leaving open sites on the beads surface.

3. With higher concentrations (10 mg/mL) beads are totally blocked, i.e. ‘masked’.
When using the NH$_2$-BeadMicroArray for immobilizing proteins the adsorption of BSA over the NH$_2$-microspheres represents a problem. To minimize the adsorption of BSA over the NH$_2$-microspheres, low concentration (e.g., 50 µg/mL) of blocking solution should be used. This should minimize the ‘masking’ of NH$_2$-microbeads while still ensuring complete adsorption over the PDMS surface. Using a BSA solution with lower concentration does not represent a problem for complete coating of the PDMS surface. In the previous Chapter, for instance, a protein solution of 50 µg/mL was adsorbed over a PDMS stamp and used for µCP, leading to a uniform print.

6.3.4.2 Selective protein immobilization and biorecognition

The propensity of BSA to adsorb over the NH$_2$-microbeads suggests that biorecognition-rather than spontaneous adsorption-should be chosen as the mean of protein immobilization. Beads terminated with biotin, for instance, would prevent unspecific adsorption of BSA and, concurrently, provide an anchorage point for selective immobilization of avidin (or streptavidin). This is the method that was employed in the present work. Biotin was first covalently attached to the NH$_2$-microspheres and then used for the fabrication of a biotin-BeadMicroArray.

6.3.4.2.1 Microsphere biotinylation

Biotin-coated polystyrene microspheres are commercially available, but-as discussed earlier in the Chapter-in this work these types of microbeads are unsuitable because of their low density. Biotin-terminated glass microspheres can be custom-synthesized by some manufacturers. Biotin terminated melamine microspheres are not commercially available and needed to be fabricated.

Biotin can be easily attached to the amino-terminated melamine microspheres using various biotin-ligands commercially available. The ligands have an N-Hydroxysuccinimide ester (NHS) attached to the valeric acid carboxylate chain of biotin (NHS-biotin). NHS-Biotin can be used to covalently modify the amino groups in proteins and other molecules (27). The NHS terminus of NHS-Biotin reacts by nucleophilic attack of an amine to the biotin carboxylate group, releasing NHS, and forming a stable amide linkage. NHS-biotin in insoluble in aqueous environments, but its sulfo derivate (sulfo-NHS-Biotin) is water soluble. In this work, the ligand sulfo-
NHS-LC-biotin was chosen, which chemical structure and reaction with amino-terminated molecules is illustrated in Figure 6.11.

The extended spacer arm (LC) allows greater distance between the biotin rings and the modified molecule (i.e., NH$_2$-microbeads in the present work). The presence of the
spacer is particularly useful when attaching a biotin-ligand to a surface to mediate the attachment of avidin. The spacer reduces the steric hindrance among the biotin groups, maximizing their exposure to the avidin molecules in solution.

Biotin microspheres were synthesized following the manufacturer protocol, i.e., by reacting an aqueous solution of sulfo-NHS-LC-biotin with an aqueous solution of amino microspheres. After removal of the supernatant and repeated washing with ultrapure water, the beads were tested for their selective reactivity towards streptavidin (Alexa Fluor 488 labelled).

Streptavidin adsorbs non-specifically to the NH$_2$-microspheres. A simple test was performed placing a sample of NH$_2$-microspheres in a solution of streptavidin-Alexa Fluor 488. Spheres were washed well with buffer and imaged at the confocal microscope, where bright fluorescent spheres were detected (Figure not shown). Therefore, to test the biotin-coated microsphere against a control sample of amino spheres, it is necessary to block the control against non-specific adsorption, for instance using BSA (1% w/v). A sample of biotin-coated microspheres was also reacted with BSA under identical conditions. The samples were then centrifuged, the blocking solution removed, and the beads extensively washed with PBS buffer. The two samples were then reacted (separately) with a 50 µM/mL solution of streptavidin-Alexa Fluor 488 labelled. After centrifugation, removal of the protein solution and washing with buffer, the beads were dispersed in buffer and the two samples imaged at the confocal microscope. A second control (un-reacted NH$_2$-microbeads) was also imaged.

Prior to any reaction, the NH$_2$-microbeads are non-fluorescent. As expected, blocking with BSA reduces the adsorption of streptavidin over the control beads to a minimum (Figure 6.12 (a)). Differently, a highly uniform fluorescent signal is registered for the labelled streptavidin attached to the biotin-coated beads (Figure 6.12 (b)).
Figure 6.12 Streptavidin-biotin selective biorecognition on biotin beads. LSCM images of (a): control, NH$_2$-beads reacted with streptavidin-Alexa Fluor 488; and (b): biotin-microbeads after reaction with streptavidin-Alexa Fluor 488. Both samples were treated with BSA prior to the reaction with streptavidin. TR=transmission light; Ch1= Ex 488 nm/Em500-530. Scale bar in (a, left) is 10 µm and applies to all images.

This result confirms the presence of biotin groups attached to the microbeads surface. Moreover, the signal suggests that the biotin coverage is uniform. Unspecific adsorption of streptavidin over the biotin beads is precluded by the treatment with BSA, therefore the immobilization of streptavidin occurs only through biorecognition.
6.3.4.3 Biorecognition using a biotin-BeadMicroArray

A biotin-functionalised BeadMicroArray was fabricated and its activity and biospecificity tested against streptavidin-Alexa Fluor 488. For brevity, the biotin-functionalised BeadMicroArray will be further referred to as biotin-BMA.

The fabrication of a biotin-BMA platform follows the same protocol described at the beginning of the Chapter, i.e., deposition via spin coating of the biotin-coated beads in the microwells of the silicon master and then molding PDMS against this master. Biotin is a thermostable biomolecule (the biotin-streptavidin association is stable up to 112 °C (28); therefore curing at 60 °C does not pose a risk for its stability. After fabrication, a biotin-BMA platform was tested against streptavidin-Alexa Fluor 488. A control biotin-BMA (fabricated in the same way) was tested against anti-mouse IgG Alexa Fluor 546 and another un-reacted biotin-BMA was used as a second control. Before the reaction of the two platforms with the labelled proteins, each biotin-BMA (not including the second control) was blocked against unspecific protein adsorption using BSA 1 % w/v. The two samples were then separately incubated with the labelled proteins (each at the concentration of 50 µg/mL), throughout rinsed with PBS buffer and water, and then imaged with the confocal microscope with the 488 nm (Channel 1) and 543 nm (Channel 2) laser lines.

Prior to any reaction, a biotin-BMA platform imaged with the confocal microscope is non-fluorescent (Figure 6.13 (a)). A small signal is detected corresponding to the biotin-coated beads, which is due to the auto-fluorescence of the melamine polymer (as assessed by a separate control). When the biotin-BMA is exposed to a solution of anti-mouse IgG Alexa Fluor 546 labelled, no recognition occurs (Figure 6.13 (b)). In contrast, when the biotin-BMA is exposed to a solution of streptavidin Alexa Fluor 488, a strong signal is detected (Figure 6.13 (c)). The small fluorescence outside the BeadMicroArray area is probably due to some incomplete BSA coating.
Figure 6.13 Selective streptavidin recognition on a biotin-BeadMicroArray. LSCM of (a): control, i.e., unreacted biotin-BMA; (b): biotin-BMA after reaction with anti-mouse IgG-Alexa Fluor 546; and (c): biotin-BMA after reaction with streptavidin-Alexa Fluor 488. All samples were treated with BSA prior to the biorecognition experiment. TR=transmission light; Ch1= Ex 488 nm/Em500-530. Ch2= Ex 543 nm/Em550-655 Scale bar (in a, left) is 10 μm and applies to all images.
These results show that the biotin functionality in the microspheres is preserved during the fabrication process. This is illustrated by the retained capacity of selectively binding streptavidin; blocking with BSA prior to incubating the biotin-BMA platform with streptavidin excludes that the latter is immobilized over the microspheres through non-specific adsorption.

The fluorescent array in Figure 6.13 (c) has an excellent signal-to-noise ratio (S/N=10); moreover, a code is clearly visible, thus demonstrating the encoding capability of the BeadMicroArray platform.

### 6.3.5 Conclusions

The BeadMicroArray platform functionalized with biotin-microbeads is a versatile platform for the selective immobilization of proteins. Validation of the platform was performed using labelled streptavidin and labelled anti-mouse IgG, only the first one being selectively recognized by the biotin-BeadMicroArray. For its use as a general platform for protein detection, avidin can be used as a homobifunctional linker to link biotinylated proteins. Alternatively, streptavidin or avidin microspheres can be directly used during fabrication of the array and used for recognizing biotinylated proteins. The fabrication of the BeadMicroArray does not pose a risk for the bioactivity of these proteins, which denature at 75° C (unliganded protein (28)). When selective immobilization of proteins is sought, BSA blocking is required to avoid protein non-specific adsorption over the PDMS surface. BSA blocking should be performed using a low concentration and after having modified the microbeads with the biotin-ligand, since BSA adsorbs non-specifically to the unmodified amino-terminated beads.

Although the use of biotinylated microspheres is advantageous and effective, other ligands can be attached to the spheres and used for the biorecognition of desired targets. Also, in the present work the platform was demonstrated for its capability of detecting proteins, but it is reasonable to believe that it can be used to detect other molecules as well, such as peptides and carbohydrates. Beads with a selected chemical functionality (e.g., amino, carboxyl, aldheide, epoxy etc.) can be employed during the fabrication process, thus providing a platform that has no limitation on the type of chemistry that can be employed for the binding.
Finally, the BeadMicroArray platform is a spatially addressable, encoded microarray. It adds a new capability for the spatial immobilization of proteins, since every single microarray is characterized by a unique code which position is known \textit{a priori}, being the array a replica of a lithographically-made master.
7 **CONCLUSIONS AND FUTURE WORK**

7.1 **CONCLUSIONS**

Three novel micropatterning techniques for the spatial immobilization of proteins have been developed and reported in this Thesis. All these techniques allow the immobilization of proteins in micro-sized patterns with bioactivity retention. Also, in these techniques, protein non-specific adsorption is minimized, therefore leading to patterns of good S/N, which improves the read-out of the patterned substrate.

The techniques developed are based on three different micropatterning approaches, namely 1) direct-writing UV laser microablation (‘proLAB’); 2) a novel microcontact printing method (‘μCPTA’); and 3) a replica molding method combined with microbead self-assembly (‘BeadMicroArray’). The first two techniques, proLAB and μCPTA, are an implementation of existing techniques (laser ablation and μCP, respectively), whereas the third, i.e., the BeadMicroArray, is a totally new technique.

7.1.1 **The ProLAB micropatterning technique**

Laser microablation is an established technique used to create profiled patterns for the immobilization of proteins. Commonly, polymers sensitive to the deep-UV region are patterned with the use of expensive, specialized equipment (e.g., deep-UV, e-beam or ion-beam lithography). The alternative is the use of UV sensitive polymers, which are fluorescent in this region, thus with the drawback of generating patterns over a substrate that is auto-fluorescent, which is a serious problem if the protein detection method is fluorescently-based.

In the present Thesis, this problem is solved with the introduction of the proLAB micropatterning technique, which uses a UV laser to ablate a bilayer substrate, formed of a UV-sensitive layer (Au) over a protein-adsorbent layer (PMMA). The substrate is first blocked with BSA and then microablated with a UV-laser. The instrument employed for the ablation is the LaserScissors®, which is a microscope equipped with a UV laser used in biology laboratories for cell and tissue microdissection.
Patterns in the form of microchannels as small as 3 µm can be easily fabricated and protein selectively adsorbed. Protein bioactivity is preserved during immobilization as demonstrated by the capacity of a labelled antibody to recognize selectively its antigen. Also, the direct-writing method can be used for the fabrication of a microarray where the information is encoded in each area in the form of a bar-code. To demonstrate this application, a pattern carrying four areas was fabricated, each characterized by a different code. Three different IgGs were immobilized in each area, and a control protein (BSA) was immobilized in the fourth one. The bar-coded array was then flushed with a labelled secondary antibody, which reacted exclusively with the corresponding primary antibody.

When ablating the Au/PMMA bilayer, patterns with characteristic surface hydrophobicity and nanotopography are formed, which appear to induce specific adsorption of proteins. When ablating using the laser at its full power, the ablated microchannel presents an inner hydrophobic nanochannel about 30 nm deep, where proteins preferentially adsorb. This ‘core’ nanochannel gradually disappears as the laser power is reduced, to a point where it is not formed anymore, which is reflected in a decrease of protein adsorption in this area.

While ablating the Au/PMMA bilayer, gold particles in the form of regular nanospheres are formed. Since the sensitivity of proteins to surface nanotopography is not well understood, the relationship between protein relative adsorption and nanoparticle size was investigated by using a calibrated SEM micrograph and the correspondent LSCM image after the adsorption of a labelled protein. The intensity integral calculated by the confocal software (indirectly related to the protein density) was found independent of the area of the particles. Therefore, for the size range that was investigated (170-400 nm), protein relative adsorption was found independent on nanosphere size.

### 7.1.2 Microcontact printing trapping air (µCPTA)

Microcontact printing is an established technique for printing proteins. The technique uses a PDMS stamp, fabricated by replica molding. When the mold contains features of high aspect ratio, it is prone to collapsing during stamping, which leads to poor quality printing, as well as printing in undesired areas. In this Thesis, this limitation of µCP was exploited, rather then avoided, by using a PDMS mold of pyramid-shaped posts of high
aspect-ratio organized in an array format. When the posts are closely packed, and the stamp is placed over a flat surface and allowed to collapse, a large air gap is formed around the entire array. Each post is in contact with the surface, but the area between them is not. When the stamp is inked with a protein solution, the result is the printing of a protein microarray which has a remarkably high signal-to-noise and high homogeneity. The technique allows to pattern proteins in the form of a spot (about 50 µm in diameter or less) having, in its interior, an array of micro-patterns. This way, an ‘array of arrays’ can be easily fabricated. Each spot generated with the µCPTA technique is formed of an array of elements which have similar signal-to-noise and standard deviation. Therefore, the elements of the array can be used as independent repetitions. This eliminates the need to have repetitions during printing, thus reducing the overall space of the patterned surface and reducing the amount of sample used. Protein bioactivity after µCPTA patterning was demonstrated by antigen-antibody recognition. Finally, the technique was compared with robotic spotting, compared to which µCPTA gave patterns of increased signal-to-noise and reduced variability.

7.1.3 The BeadMicroArray platform

To the best of my knowledge, only one work in the literature has used PDMS replica molding as a technique for transferring a material inside a PDMS mold. In this Thesis, for the first time, microbeads were immobilized at the tip of the post of a PDMS mold during the replication process, resulting in an array of ‘beads-on-post’. The technique uses a silicon master formed of V-shaped well arranged in an array format, and the beads are deposited inside the wells via spin-coating. The number of beads deposited per well depends on the relative dimension of the two. Conditions were optimized to obtain arrays of single beads, and about half the array filled with beads. Since bead deposition during spin-coating is totally random, a different geometric code is created every time a bead-array is fabricated. After PDMS replication, this leads to a platform having as many arrays as they were in the master, each characterized by a unique code. Thus, an encoded microarray is fabricated. During fabrication, beads of any functionality can be used, therefore leading to a platform which can be used for the selective immobilization of proteins through a variety of cross-linking reactions. In the research reported in this Thesis, amino-terminated microbeads were used and their reactivity after the fabrication process was demonstrated. The NH₂-BeadMicroArray
platform was then used for protein immobilization, using the biotin-avidin pairing system as the immobilization method. Finally, the BeadMicroArray platform with biotin-terminated microbeads was used for selectively recognize labelled streptavidin over another labelled protein (an antibody). The platform showed a remarkable level of selectively which, together with an efficient blocking towards protein non-specific adsorption, lead to a read-out characterized by a very good signal-to-noise. Also, after recognition, a code was clearly visible, therefore confirming the encoding capacity of this unique microarray. Differently to other encoding system, the one here reported is not based on fluorescence; therefore the number of codes that can be created is limited only by the number of different combinations that can be created for a given array using a certain number of beads. This is an important advantage over other encoding systems.

Finally, although in the work reported in this Thesis the BeadMicroArray was applied to protein patterning, it is conceivable that this technique can be applied to the immobilization of any biomolecule, including DNA and peptides.

7.2 FUTURE WORK

7.2.1 Improvements to the proLAB technique

Laser ablation of the Au/PMMA bilayer generates a pattern with distinct nanotopography and surface chemistry. While nanotopography could be characterized with SEM and AFM, the precise chemical nature of the microchannel originated after ablation could not be characterized. It appears from AFM and LSCM imaging that the channel is composed of three distinct areas: the edges, a core channel, and an area in between the two. There is AFM evidence to say that the core channel is hydrophobic, which is confirmed by the preference of the proteins to adsorb in this area. Therefore, an accurate chemical analysis should be performed, such as XPS, ToF-SIMS analysis or Chemical Force Microscopy (CFM). Also, other polymers different from PMMA could be used, such as PtBMA, to create microchannels of different surface chemistry, and test them for their suitability for patterning different types of proteins. Ultimately, the best conditions in terms of materials and ablation conditions could be found, so to maximize the adsorption inside the microchannels of different types of proteins.
7.2.2 Improvement to the microcontact printing trapping air (µCPTA) technique

In general, µCP techniques work well on hydrophilic substrates when an untreated, hydrophobic PDMS stamp is used. In this work, µCPTA was demonstrated using a glass substrate; protein transfer was indeed excellent on glass (which, in contrast, failed over a hydrophobic surface like polystyrene), but when the patterned substrate was exposed to repeated rinsing, de-sorption was an obvious problem. Covalent binding would solve this problem, resulting in more stable patterns. Hydrophilic substrates like glass are also convenient since these surfaces repel protein adsorption from solution, a convenient feature when the patterned substrate is further processes, like for selective recognition. The solution is therefore the use of a hydrophilic substrate where proteins can be attached via covalent binding, such as a carboxyl-terminate surface that concurrently prevents protein adsorption from solution. For example, a gold surface with a mixed monolayer of COOH- and OH-terminated SAMs would be suitable, or a PET (poly(ethylene terephthalate)) film derivatized with carboxyl acid groups.

In the work reported in this Thesis µCPTA was demonstrated with a stamp having pyramid-shaped posts, which lead to star-shaped features. Rectangular posts with a square base or cylindrical posts are likely to result in square and circular features, respectively, a more regular shape that would lead to more regular arrays. This would further decrease the variability of the patterns forming the array and would improve the microarray read-out.

7.2.3 Improvements to the BeadMicroArray platform

Presently this technique when applied to the immobilizing proteins requires blocking the PDMS surface against protein non-specific adsorption, which is favoured by the hydrophobic nature of PDMS. To solve this problem, an alternative hydrophilic polymer compatible with replica molding could be used, i.e., a material that is liquid before curing, solidifies during curing to form a rubber, but can also be easily peeled off from the silicon master at the end of the process. As discussed below, finding such material is not trivial.
Hydrophilic rubbers don’t exist; their hydrophobicity is a consequence of their chemical structure. A hydrophilic, flexible material that could be used instead of PDMS is a hydrogel. Hydrogels belong to a class of gels particularly popular in tissue engineering applications, because their water content and mechanical properties closely resemble that of tissues in the body. This type of material, though, would introduce two, more important problems in the BeadMicroArray platform: the resulting platform would be mechanically unstable and the porous nature of the gel would adsorb liquid form its surface. Therefore, hydrogels are less suitable alternative to PDMS. The only real alternative to PDMS would be a co-polymer formed of PDMS and a hydrophilic polymer like poly(vinyl alcohol), PVA. This polymer is biocompatible, but it is not a rubber. If a co-polymer with PDMS was synthesized, though, a rubber with PVA components could be obtained, which may create a surface less prone to protein adsorption. This work would require extensive knowledge of polymer chemistry.

Finally, in the research reported in this Thesis, the BeadMicroArray platform was applied to protein immobilization, specifically using amino- and biotin-terminated microbeads. The platform could be fabricated using other types of microbeads (e.g., avidin-coated) and used for immobilizing others types of biomolecules, such as DNA, thus providing a new DNA microarray platform with a versatile encoding capability.


121. Nicolau DV. Biomolecular adsorption database. URL: www.bionanoeng.com/bad


