Patterned and switchable surfaces for biomaterial applications

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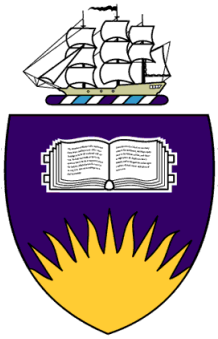

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"*If at first, the idea is not absurd, then there is no hope for it.*"

Albert Einstein

"Science is not formal logic–it needs the free play of the mind in as great a degree as any other creative art."

Max Born

"One had to be a Newton to notice that the moon is falling, when everyone sees that it doesn't fall."

Paul Valéry

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SUMMARY

The interactions of biomolecules and cells at solid-liquid interfaces play a pivotal role in a range of biomedical applications and have hence been studied in detail. An improved understanding of these interactions results in the ability to manipulate biomolecules and concurrently cells spatially and temporally at surfaces with high precision. Spatial control can be achieved using patterned surface chemistries whilst temporal control is achieved by switchable surfaces. The combination of these two surface properties offers unprecedented control over the behaviour of biomolecules and cells at the solid-liquid interface. This is particularly relevant for cell microarray applications, where a range of biological processes must be duly controlled in order to maximise the efficiency and throughput of these devices. Of particular interest are transfected cell microarrays (TCMs), which significantly widen the scope of microarray genomic analysis by enabling the high-throughput analysis of gene function within living cells

Initially, this thesis focuses on the spatially controlled, electro-stimulated adsorption and desorption of DNA. Surface modification of a silicon chip with an allylamine plasma polymer (ALAPP) layer resulted in a surface that supported DNA adsorption and sustained cell attachment. Subsequent high density grafting of poly(ethylene glycol) (PEG) formed a layer resistant to biomolecule adsorption and cell attachment. PEG grafted surfaces also showed significantly reduced attachment of DNA with an equilibrium binding constant of 23 ml/mg as compared with 1600 ml/mg for ALAPP modified surfaces. Moreover, both hydrophobic and electrostatic interactions were shown to contribute to the binding of DNA to ALAPP. Spatial control over the surface chemistry was achieved using excimer laser ablation of the PEG coating which enabled the production of patterns of re-exposed ALAPP with

high resolution. Preferential electro-stimulated adsorption of DNA to the ALAPP regions and subsequent desorption by the application of a negative bias was observed. Furthermore, this approach was investigated for TCM applications. Cell culture experiments demonstrated efficient and controlled transfection of cells. Electro-stimulated desorption of DNA was shown to yield enhanced solid phase transfection efficiencies with values of up to 30%. The ability to spatially control DNA adsorption combined with the ability to control the binding and release of DNA by application of a controlled voltage enables an advanced level of control over DNA bioactivity on solid substrates and lends itself to biochip applications.

As an alternative approach to surface patterning, the fabrication and characterisation of chemical patterns using a technique that can be readily integrated with methods currently used for the formation of microarrays is also presented. Here, phenylazide modified polymers were printed onto low fouling ALAPP-PEG modified surfaces. UV irradiation of these polymer arrays resulted in the crosslinking of the polymer spots and their covalent attachment to the surface. Cell attachment was shown to follow the patterned surface chemistry. Due to the use of a microarray contact printer it was easily possible to deposit DNA on top of the polymer microarray spots. A transfected cell microarray was generated in this way, demonstrating the ability to limit cell attachment to specific regions and the suitability of this approach for high density cell assays. In order to allow for the high-throughput characterisation of the resultant polymer microarrays, surface plasmon resonance imaging was utilised to study the adsorption and desorption of bovine serum albumin, collagen and fibronectin. This analysis enabled insights into the underlying mechanisms of cell attachment to the polymers studied. For the system analysed here, electrostatic interactions were shown to dominate cellular behaviour.

DECLARATION

I certify that this thesis does not incorporate without acknowledgment any material previously submitted for a degree or diploma in any university; and that to the best of my knowledge and belief it does not contain any material previously published or written by another person except where due reference is made in the text.

Andrew Leslie Hook

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LIST OF PUBLICATIONS

Papers arising from Chapter 1

1. Hook, A.L., N. Voelcker, and H. Thissen, *Patterned and switchable surfaces for biomolecular manipulation.* Acta Biomaterialia, **2008**, *Under review*.
2. Hook, A.L., N. Voelcker, and H. Thissen, *Surface manipulation of biomolecules for cell microarray applications.* TRENDS in Biotechnology, **2006**, *24(10)*, 471-477.

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3. Hook, A.L., H. Thissen, J.P. Hayes, and N.H. Voelcker, *Spatially controlled electro-stimulated DNA adsorption and desorption for biochip applications.* Biosensors and Bioelectronics, **2005**, *21(11)*, 2137-2145.
4. Hook, A.L., H. Thissen, J.P. Hayes, and N. Voelcker, *A platform for the advanced spatial and temporal control of biomolecules.* Proceedings of SPIE, **2006**, *6413(64130C)*, 1-11.
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I

CHAPTER 1. INTRODUCTION

The content of this chapter is based upon references [1, 2].

Advanced biodevices that are able to control the behaviour of biomolecules at surfaces in both space and time are promising tools for elucidating solutions to many biologically based problems and are of particular interest to combat physiological disorders. Biomolecules of interest include proteins and shorter peptide chains, deoxyribonucleic acid (DNA) (for a full glossary of abbreviations see Appendix 1), ribonucleic acid (RNA), oligonucleotides (oligonucleotides are short (2-50) bp of typically single stranded DNA), lipids and polysaccharides, as well as larger assemblies of these biomolecules, in particular living cells. Examples of such devices can be found in microarray technology, in 'smart' drug delivery, biosensing, bioelectronics and tissue engineering [3-8]. The development of a number of high-resolution two dimensional (2D) and three dimensional (3D) patterning techniques coupled with functional surface chemistry has enabled the formation of surfaces that offer stringent control over the adsorption of biomolecules and cells in space. Furthermore, the development of switchable surfaces that are able to respond to a particular signal to switch between disparate properties, such as hydrophobic/hydrophilic, positive/negative or swollen/collapsed, has added a new dimension to biomolecule manipulation. Individually, these processes have enabled the production of a number of advanced biodevices. Recently, these processes have been combined, producing devices that are able to control biomolecules and cells in both space and time, offering an unprecedented ability to manipulate biomolecular behaviour.

In order to manipulate biomolecules at surfaces, a thorough understanding of their behaviour at solid-liquid interfaces is required. Biomolecules differ substantially from their synthetically produced polymeric counterparts of similar molecular weight due to the narrow dispersity in structure and size for the former. This results in unique and predictable adsorption behaviour, providing a unique opportunity for highly resolved control over these biomolecules at an interface. Indeed, in biological systems highly resolved spatial and temporal control of biomolecules is a critical requirement for the phenomenon of life. The thermodynamic and kinetic driving forces to permit this control are programmed into the sequence and 3D structure of biomolecules. An ability to better understand these driving forces would permit an increased capability to mimic *in vivo* biomolecular manipulation.

This chapter summarises the current knowledge on the underlying principles governing both DNA and protein adsorption to surfaces and how protein adsorption can be applied to manipulating cells at surfaces. Furthermore, the manner by which these principles have been applied in recent years to pattern biomolecules on surfaces and also to control their adsorption and desorption in time is discussed. The chapter also includes an outline of the various techniques used to form patterned and switchable surfaces. The particular focus here has been on cases where truly advanced biomolecule manipulation is achieved in both space and time.

1.1. Surface manipulation of biomolecules and cells

The ability to manipulate biomolecules at the solid/liquid interface requires a sound knowledge of how biomolecules behave in such an environment. The manipulation of biomolecules is significantly different from the manipulation of smaller molecules or synthetic polymers. Weak forces such as hydrophobic

interactions are able to play a significant role given the ability of these biomolecules to form multivalent interactions. The size of these molecules also plays a considerable role in regard to their behaviour at surfaces. For example, larger molecules tend to have a lower rate of surface adsorption when compared with smaller molecules which adsorb, desorb and diffuse more readily from and to the surface [9]. Both DNA and proteins have distinct characteristics that must be understood in order to effectively manipulate these molecules on surfaces. Cell-surface interactions can also be controlled effectively via the control of biomolecule-surface interactions since most cell-surface interactions are mediated by protein adsorbed on surfaces.

1.1.1. Principles of surface-biomolecular interactions

Although distinctive properties of specific biomolecules greatly influence their surface adsorption events, generally, hydrophobic interactions and the multivalent effect are key factors that govern the adsorption behaviour of many biomolecules at surfaces. Understanding these principles can concurrently lead to explanations of biomolecule-surface interactions.

1.1.1.1. The hydrophobic interaction

One of the key interactions for all biomolecule surface adsorption is the hydrophobic interaction. This results when hydrophobic domains or moieties are present on both the surface and the biomolecule of interest. Thermodynamically, the driving force for the adsorption of biomolecules through formation of hydrophobic interactions is entropic gain due to the disordering of hydrophilic solvent molecules that must otherwise become ordered at a hydrophilic/hydrophobic interface. Thus, the solvent has a vital role in the formation of these interactions and in the presence

of a hydrophobic solvent, where there is no entropic gain in forming a hydrophobic interaction, these interactions are strongly reduced [10]. In water, any biomolecules less polar than water or containing regions less polar than water will be driven to adsorb to hydrophobic surfaces by hydrophobic interactions [11].

1.1.1.2. The multivalent effect

Generally, interactions of biomolecules with surfaces are based upon weak forces. Thus, a key factor to ensure that any biomolecule remains adsorbed to the surface is the multivalent effect, whereupon many small bonds form and the combination of these many bonds leads to the formation of an overall strong interaction. The multivalent effect is thermodynamically favourable due to the increase in entropy introduced when a single large molecule adsorbs to the surface, displacing multiple smaller molecules. This effect pertains particularly to biomolecules due to their larger size. It is on the basis of the multivalent effect that surface diffusion can be explained. As any biomolecule is held to the surface by a number of weak interactions, at any time some of these bonds can break and reform at another location, however, provided enough bonds remain intact the molecule itself will not break from the surface. In such a manner the biomolecule is able to 'roll' along the surface where the molecule only partially adsorbs and desorbs [12]. The introduction of stronger biomolecule-surface interactions would, thus, also decrease the rate of surface diffusion due to a decreased rate of bond breakage [13].

1.1.2. Surface manipulation of DNA

In applications such as DNA microarrays, DNA based-biosensors and transfected cell microarrays (TCMs) [1, 14-17], the adsorption or desorption of DNA to or from a surface is required. The adsorption of DNA to a surface is governed by two forces

associated with the functional groups of DNA; electrostatic forces associated with the negative charge of the phosphate groups and hydrophobic forces associated with interactions of the DNA base pairs [18, 19], although hydrogen bond formation also plays a certain role in DNA surface interactions in aqueous conditions [20]. Unlike synthetic polyelectrolytes, which generally adopt a loop-train conformation when adsorbed to a surface due to their flexible 'thread-like' nature [21], double-stranded DNA (dsDNA) can be considered as a rigid rod (Figure 1.1B) with a nitrogenous core and a phosphate and pentose sugar exterior (Figure 1.1A) such that it generally lies flat to a surface upon adsorption [22]. Conversely, although more ordered and rigid than synthetic polyelectrolytes, single-stranded DNA (ssDNA) can be considered as a flexible thread, not nearly as rigid as dsDNA, with the nitrogenous bases readily exposed (Figure 1.1C). Thus, hydrophobic interactions play a more significant role with the adsorption of ssDNA as compared with dsDNA [22].

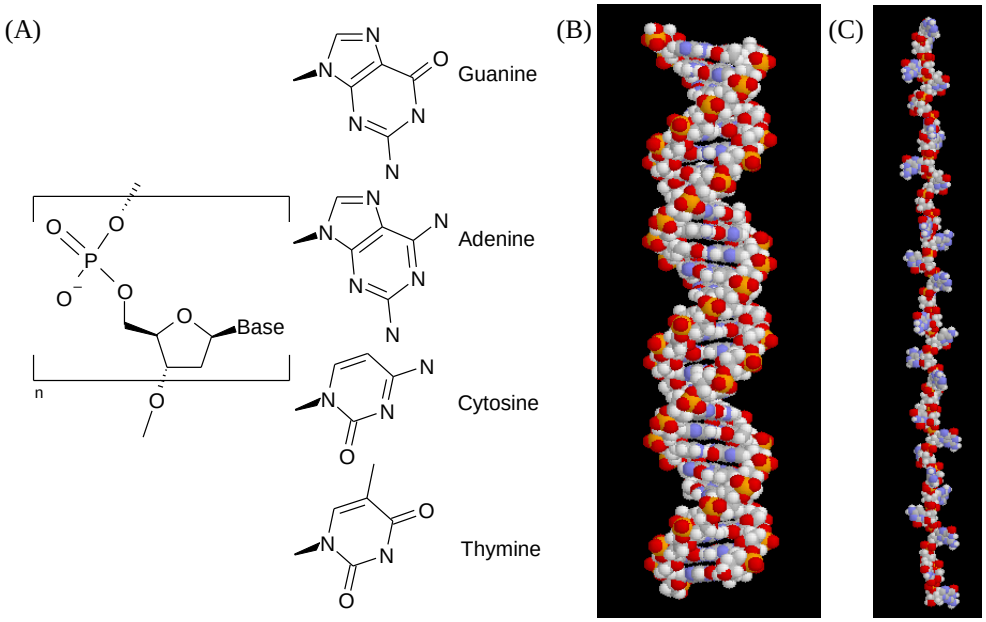

Figure 1.1. *Structure of DNA. (A) The chemical structure of DNA nucleotides, (B) three dimensional space filling model of B-type helical dsDNA, (C) three dimensional space filling model of ssDNA.*

In general, higher salt concentrations and lower pH increase the propensity for DNA adsorption and likewise, low salt concentration and high pH both increase the propensity for DNA desorption [20, 23, 24]. This is a result of two effects. First, under high salt concentration or low pH the strength of the DNA-surface electrostatic interactions can be increased by enhancing the cationic character of a surface and dampening the anionic character of DNA, which allows the DNA to proceed close enough to the surface for hydrophobic interactions to occur. Second, and more commonly observed with the use of cationic ions with a high valency of typically +3 or more, individual DNA strands are compacted due to neutralisation of repulsive electrostatic forces between adjacent phosphate groups, resulting in more compact DNA molecules and, therefore, the scope for higher surface coverage of DNA (Γ^{DNA}) [25, 26].

Electrostatic interactions with DNA have been the primary focus in studies where the adsorption of DNA and its manipulation was desired. The formation of positively charged surfaces is often employed for DNA adsorption experiments. This is commonly achieved by the production of amine rich surfaces, which are typically protonated at neutral pH, and have been shown to increase the Γ^{DNA} [27]. The ease of formation of strong DNA-surface interactions using aminated surfaces where hybridisation can still proceed [28] makes this an attractive approach for microarray applications as opposed to covalently immobilising DNA. A common strategy for the formation of aminated surfaces is silanisation of glass [9, 24, 28-30]. Aminated surfaces have also been produced by the adsorption of cationic molecules such as poly(ethyleneimine) (PEI) [31] or poly(L-lysine) (PLL) [32] and plasma polymerisation [33-37]. For a comprehensive review of plasma polymerisation see reference [38]. Interestingly, Saoudi et al., [27] reported the adsorption of DNA to

aminated polypyrrole silica particles, which had a near-zero surface charge despite the presence of protonated amine groups. The positive charges are compensated by anionic silanol groups. This study suggests that isolated positively charged groups and not a net positive surface charge are sufficient to stimulate DNA adsorption. Lemeshko et al., [28] investigated simplifying DNA microarray formation by adsorbing DNA probes to a surface utilising the electronegative nature of DNA for formation of electrostatic interactions with a positively charged 3-aminopropyltrimethoxysilane modified surface instead of by covalent linkage. A densely packed single-stranded oligonucleotide layer was successfully adsorbed to this surface, where the ssDNA oligomers were adsorbed side-on on the surface, and was used for subsequent hybridisation experiments that confirmed the accessibility of the adsorbed DNA probes for the formation of base pairs with complementary target DNA strand. Interestingly, asymmetric dissociation and DNAse digestion was observed for dsDNA formed in the manner described, whereupon, the hybridised target DNA strands dissociated quicker and were more heavily digested than the initial electrostatically bound probes. This suggests that a typical DNA helix is not formed between these two strands upon hybridisation.

More recently, the polyelectrolytic nature of DNA has been utilised for formation of multilayered films consisting of alternating layers of DNA and cationic polyelectrolytes [32, 39-41]. One polycation commonly used is PEI, which has been used to allow the adsorption of plasmid DNA to poly(lactic acid) (PLA) particles [42]. Yamauchi et al., [41] utilised this strategy to attain a very high Γ^{DNA} for TCM applications. Layer-by-layer assembly of PEI and plasmid DNA was utilised for formation of an electrode with a Γ^{DNA} of 0.6 $\mu g/cm^2$ that was subsequently used for the transfection of cells adherent on the electrode.

For some applications it has been desirable to adsorb DNA to negatively charged surfaces. This has been the case for atomic force microscopy (AFM) experiments with DNA, which commonly require adsorption of DNA to atomically flat, hydrophilic mica and also for adsorption to silica, which is an important and ubiquitous material for many biodevices [20]. The electrostatic repulsion between DNA and mica or other negatively charged surfaces or particles can be overcome, enabling DNA adsorption, in the presence of a divalent cation such as Mg^{2+}, which can act as bridging cations [20, 23, 43]. Generally, trivalent cations are more effective than divalent cations, which in turn are more effective than monovalent cations at enabling DNA adsorption. Hansma et al., [44] used AFM to study the effect of the type of divalent cation on binding of dsDNA of lengths from 79-1057 bp to mica. High amounts of DNA adsorbed to mica in the presence of Ni, Co and Zn ions, however, weak adsorption was seen with Mn, Hg and Cd ions. This effect was explained in terms of the structure of mica and how each type of ion interacted with the mica as opposed to a specific ion-DNA interaction, suggesting that the type of ion most suitable at enhancing DNA adsorption is dependant on the particular substratum surface.

1.1.3. Surface manipulation of proteins and cells

Many biodevice applications, including tissue engineering, cell microarrays and implants [4, 45-47], require the ability to manipulate proteins, and concurrently cells, at interfaces. The fundamental nature of how proteins behave at surfaces depends largely upon their primary structure, that is, the sequence of amino acids making up the protein. There are four main properties of amino acids that influence the behaviour of proteins; polar, non-polar, negatively charged and positively charged. It

is no surprise then that the main two interactions of proteins with surfaces, like in the case of DNA, are electrostatic and hydrophobic interactions.

As proteins function predominantly within aqueous environments, with the exception of membrane bound proteins, proteins try to minimise the entropic penalty of interactions with water with hydrophobic domains by shielding as many hydrophobic amino acids within the protein core whilst arranging the hydrophilic amino acids on the protein surface. However, this 'phase separation' is not always complete, particularly within smaller proteins that have a larger surface area to volume ratio. Thus, hydrophobic domains often exist on the surface of proteins, and those readily adsorb to hydrophobic surfaces even in the presence of electrostatic repulsion due to the large increase in entropy associated with surface de-solvation and the minimisation of polar/non-polar interfaces [11]. This thermodynamically favourable process is the driving force for protein adsorption. However, adsorption to a surface, particularly a hydrophobic surface, can lead to a rearrangement of hydrophobic domains within the centre of proteins to enable the formation of hydrophobic contacts between those domains and the surface. Thus, although highly hydrophilic surfaces will generally reduce protein adsorption, hydrophobic surfaces or hydrophobic patches on otherwise hydrophilic surfaces can cause the rearrangement of proteins resulting in their denaturation and exposure of previously buried hydrophobic residues. This protein rearrangement upon surface adsorption is driven by an increase in entropy due to the destabilisation of ordered protein domains, such as α-helices and β-sheets [11].

Elwing et al., [48] investigated the adsorption of human γ-globulin, human fibrinogen and lysozyme onto a surface with a wettability gradient. This surface was prepared using silanes onto doped silicon to form a wettability gradient with contact

angles ranging from 20-80˚. Significantly, for all proteins a higher surface coverage ($\Gamma^{protein}$) was attained at the hydrophobic end [48]. However, significant amounts of protein adsorption were still observed towards the hydrophilic end of the gradient, suggesting that although surface wettability plays a significant role in protein adsorption it is still not the only force at play. As a variant of this work, Tilton et al., [10] investigated the adsorption of ribonuclease A onto polystyrene (PS) in different alcohol co-solvents of different polarity. Decreasing adsorption propensity was demonstrated in solvents of decreasing polarity, which can be explained in terms of the minimisation of the driving force of protein adsorption [10].

As proteins are usually not geometrically symmetric, the orientation of adsorbed protein can impact upon its $\Gamma^{protein}$. For example, if a protein had an oblong shape such that there was a significant difference between the projected areas of the side-on and end-on orientation, then if all proteins were able to adsorb in an end-on orientation, a greater $\Gamma^{protein}$ would be attained as compared with an all side-on attachment [10]. The orientation of adsorbed proteins impacts not only upon $\Gamma^{protein}$. Surface orientation is of even greater importance for protein activity. Even if a high $\Gamma^{protein}$ is attained, most applications for protein adsorption are not viable if the adsorbed protein is not bioactive. For this reason, various approaches have been developed to control the orientation of adsorbed proteins. This is often achieved by modifying proteins at specific points on the protein exterior with molecular tethers or tags. Common approaches include the use of poly-histidine tagged protein, which forms a complex with surface bound nickel ions [49] and biotin labelled proteins that can form a strong biotin-avidin bridge with avidin functionalised surfaces [50]. A comprehensive review of methods to control protein orientation is available to the interested reader [51].

Living cells exist in nature within the extracellular matrix (ECM), which is a network of biomolecules forming the framework that cells attach to and are supported by. This matrix is composed largely of polysaccharides and various proteins; and it is these proteins, in particular collagen, fibronectin and vitronectin, that cells use to attach to the ECM. Thus, the adhesion of cells to a tissue is mediated by proteins that are already present on that tissue or that the cell produces itself. The same is true *in vitro* and, therefore, the mechanisms used to control protein adsorption, which have been discussed, can equally be applied to cell attachment.

Manipulating cell attachment has attracted much interest and is important for a wide range of biodevice applications. Research has been focussed on producing surfaces that support or resist cell growth, and more specifically, surfaces that either switch between an adherent and a non-adherent surface on demand or have the ability to direct cell growth to localised areas.

One of the most effective methods for promoting cell attachment is the use of extracellular proteins and in particular the Arg-Gly-Asp (RGD) integrin binding peptide based on a cell adhesion mediating site of fibronectin [33, 52-54]. This method is very effective as it utilises the mechanisms by which cells attach to surfaces within natural systems. Various other materials and surface chemistries, such as tissue culture PS and polyurethane, support cell attachment but the use of ECM proteins is unrivalled in its ability to actively promote cell attachment.

As well as chemically initiated cell attachment, the effects of topographical cues to initiate and control the attachment, proliferation, orientation and migration of cells and tissue samples on surfaces has been investigated [47, 55-62]. The ability of substratum topography to influence cell outgrowth suggests that the ECM may present both chemical and topographical signals to cells and is noteworthy in terms

of understanding phenomena like embryo development and wound healing and for the improvement of implant compatibility. Significantly, substrates with aligned grooves have been shown to orient cell growth parallel to the direction of the grooves whilst also improving cell attachment as compared with flat surfaces [57-59], enabling the formation of patterned cell growth. Of interest are studies that demonstrate the ability of micro- and nanostructures on surfaces that are smaller than the typical cell dimensions (≈ 20 μm) to influence the behaviour of cells [57, 58]. Use of cliffs, pillars and islands on surfaces have also been shown to influence cell outgrowth [60]. The mechanism behind this effect is not fully understood. In some cases, improved attachment may be due to the increased surface area of the surface. Recently, Wan et al., [63] investigated cell attachment of osteoblast-like cells (OCT-1) on microfabricated PS and PLA surfaces with hemispherical bump and pit surface features. These features had a bimodal distribution of sizes with an average diameter of 2.2 and 0.45 μm. The behaviour of the OCT-1 cells on the pit-patterned PS surface was most notable (Figure 1.2). Three interesting behavioural characteristics of cells were observed; first, cells showed the ability to be able to stride over both the 2.2 μm pits (Figure 1.2A) and the 0.45 μm pits (Figure 1.2B). Second, the more flexible pseudopods of the cells were able to enter into and grow along the walls of the 2.2 μm pits but not the 0.45 μm pits, which were evidently too small. Third, the filopodia of cells tended to grow along the ridge at the wall of the pits and as a result instigated a morphological change that allowed them to follow the curvature of the ridge (Figure 1.2C). This contact guidance phenomenon has also been observed in many other studies [57-59].

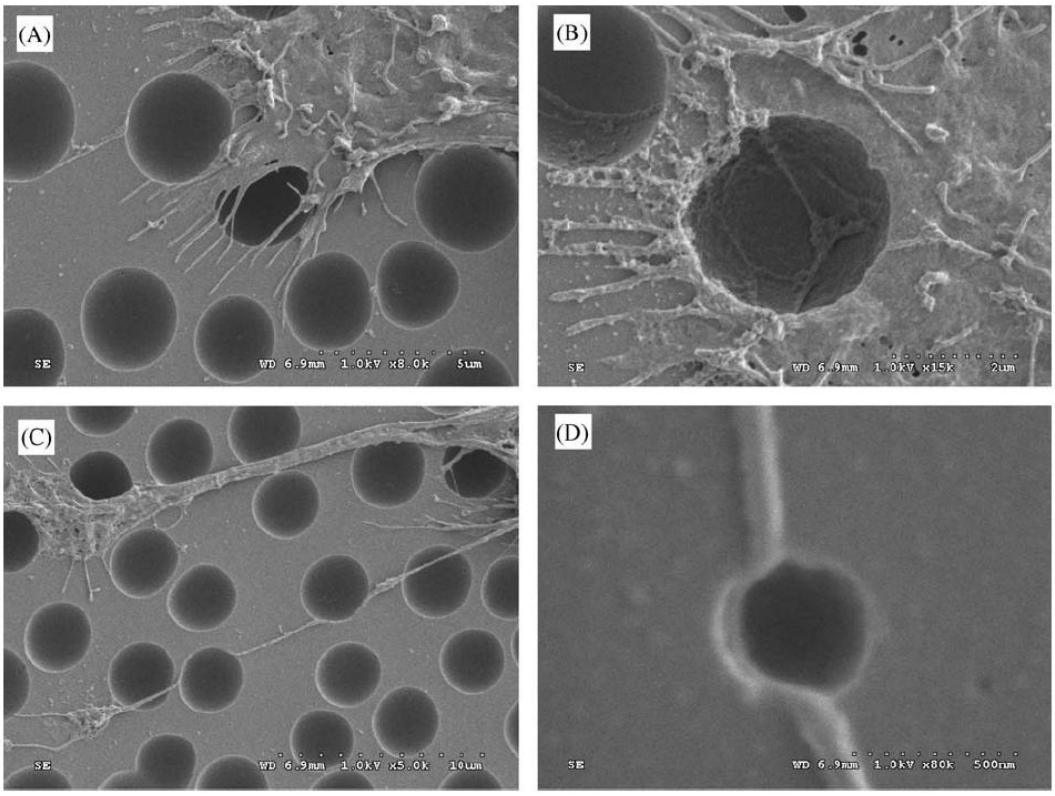

Figure 1.2. *SEM images of OCT-1 osteoblasts on different pits-patterned PS surface. (A) on micro-scale (2.2 μm) pits PS (8000x mag.) cells were observed to be able to stride over pits; (B) on micro-scale (2.2 μm) pits PS (15000x mag.) pseudopods of the cells were observed to enter into the pits and grow along the ridge at the wall of the pits instigating contact guidance of the attached cell; (C) on micro-scale (2.2 μm) pits PS (5000x mag.) filopodia of attached cells grew along the ridge at the wall of the pits instigating contact guidance of the attached cell and altering cell morphology; (D) on nano-scale pits (0.45 μm) PS (80000x mag.) cells tended to stride over these smaller features. From [63].*

Several strategies have demonstrated the ability to produce ‘low-fouling’ surfaces that resist non-specific protein adsorption, including the surface immobilisation of carbohydrates, dextrans or hydrogels [64]. However, the most common and the most effective method utilises the hydrophilic poly(ethylene glycol) (PEG) molecule either immobilised or polymerised onto the surface or other polymers or biomaterials functionalised with PEG. Three primary factors contribute to PEG’s low-fouling properties. Firstly, the hydrophilic PEG hydrogen binds extensively to water and due to its molecular structure fits well into the structure of bulk water. Protein adsorption would lead to unfavourable disruption of the hydrogen bonding. Secondly, the free energy of the polymer-water interface is minimal, decreasing the driving force of protein adsorption. Thirdly, a dense PEG brush has high volume exclusion properties due to high conformational entropy [65]. In the case of end-point grafted PEG, it has been found that the PEG coating provides an interfacial barrier that prevents proteins from interacting with the underlying substrate. Therefore, the molecular weight and interfacial graft density of PEG chains are important parameters to enable non-fouling properties of the coating [66, 67].

The production of alternative low-fouling surfaces is limited. Kleinfeld et al., [68] devised a strategy to control the attachment and outgrowth of neuronal cells on silanised silicon patterned by photolithography to have regions of alkyl silanes and amino functionalised silanes. Interestingly, the alkyl-silanes were able to resist the attachment of cells, leaving the cells to grow only on the amino functionalised regions. The low-fouling ability of this surface is presumably a result of the denaturation of secreted proteins that cells use to attach to the surface. The use of blocking proteins such as bovine serum albumin (BSA) or casein has also been used

to produce low-fouling surfaces [69]. By saturating a surface with these 'sticky' proteins, the subsequent adsorption of other proteins can be prevented.

1.2. Surface micro- and nano-patterning

The formation of micro- and nano-patterns of biomolecules on surfaces has been widely explored and enables the production of sophisticated biomaterials [6, 70, 71], the exploration of biomimetics [72], and the formation of advanced microarrays [41, 73]. Spatial control of biomolecules on solid substrate materials can be achieved using a variety of approaches to patterning. Specific strategies for the surface patterning of biomolecules include microfluidics, microcontact printing (μCP), microelectronics, photolithography, soft-lithography, laser ablation and robotic spotting [33, 74-79]. A detailed technical review of patterning techniques has recently been published [66]. Furthermore, effective spatial control of cell-surface interactions is also possible indirectly via spatially controlling biomolecule attachment to surfaces. Some of the methods that have been used with success are listed below, and a table of their advantages, disadvantages and applications is shown in Table 1.2 (see section 1.4.4.2).

1.2.1. Photolithography

Photolithography involves the irradiation of a surface by a high-energy beam, typically ultraviolet (UV) light, through a photomask. Surface alterations can include the ablation of a photoresist layer, breaking of a chemical bond resulting in the release of an attached molecule, initiation of polymerisation or initiation of formation of a chemical bond resulting in the grafting of a molecule [53, 78, 80]. Photolithography is also utilised for patterning of surfaces to create topographical

cues to control cell growth. This technique has been widely used and is able to pattern surfaces down to sub-micron dimensions, but suffers from the requirement for rigorous laboratory protocols and high setup and maintenance costs.

Photolithography has also been particularly useful for the patterning of proteins [81]. A number of approaches have been employed, but the general approach consists of coating a surface with a photoresist that is subsequently patterned by photolithography to re-expose the underlying material at specific locations. This material is functionalised, often by the use of silanes or self-assembled monolayers (SAMs) with a functional group that either adsorbs protein or is able to covalently link protein such as arylazide derivatives. The rest of the photoresist is subsequently removed and the remaining re-exposed surface is functionalised with a low-fouling material [81].

Falconnet et al., [78] produced a chemically patterned platform for spatially directed cell growth based upon the spontaneous adsorption of PLL grafted PEG to negatively charged surfaces, including oxides of niobium, titanium, silicon and indium tin as well as PS. A photoresist layer was coated onto niobium oxide coated silicon and patterned by photolithography using UV illumination. PLL-grafted-PEG functionalised with the RGD peptide was adsorbed onto the patterned surface, and the remaining photoresist was subsequently removed by washing with an organic solvent that did not disrupt the PLL-grafted-PEG layer. A pattern of functionalised PLL-grafted-PEG remained on a surface of bare niobium oxide. The bare niobium oxide was subsequently coated with non functionalised PLL-grafted-PEG, leaving a patterned surface with cell adhering regions separated by non-cell adhering regions. A pattern of human foreskin fibroblasts was successfully realised on this surface.

Otsuka et al., [80] developed a platform for spatially controlled cell growth by formation of a PEG/PLA block copolymer layer by spin coating on glass silanised with [3-(methacryloyl-oxy)-propyl]trimethoxysilane. Etching through a photomask with a nitrogen and hydrogen plasma removed the PEG/PLA layer and exposed the silanised glass. On this platform, Otsuka et al., [80] were able to demonstrate spatial control of bovine aortic endothelial cells (BAEc), which grew on the exposed glass regions that readily adsorbed proteins including ECM proteins, but not on the PEG/PLA regions where the PEG blocks were effective in resisting protein adsorption. Interestingly, rat primary hepatocytes were able to grow on both the PEG/PLA and the glass regions. Upon prior seeding and attachment of BAEc, spatial control of hepatocytes was achieved and hepatocyte spheroids grew on the etched regions carrying an underlying endothelial cell monolayer. A minimum centre-to-centre spacing between etched regions of 200 μm was also determined as sufficient to prevent bridging between colonies.

Spatially directed cell attachment has also been achieved using a vitronectin mediated, photolithographically formed, patterned *N*-(2-aminoethyl)-3-aminopropyltrimethoxysilane (EDS) and dimethyldichlorosilane (DMS) substrate. Spatially directed cell attachment was driven by the preferential adsorption of vitronectin to the EDS over the DMS [53]. The vitronectin acted as a promoter of cell growth in the regions it was adsorbed upon. This system sustained cell viability and attachment for at least 2 hr using human bone-derived cells.

Another application of photolithography is for on-chip DNA synthesis on DNA microarrays. In this technique a light source passes through a mask to direct light onto localised areas cleaving a photo-labile group and activating the site for the grafting of the next DNA nucleotide building block. By changing the photomask and

by controlling the addition of a particular nucleotide after each activation step, a high-density array of tailored sequences can be grown at the surface. This approach has also been achieved with inkjet technology, enabling probe growth without the rigorous protocols required of the photolithography strategy [82, 83]. *In situ* DNA growth enables the formation of an array with millions of probes per cm^2, however, probes are limited to 25 bases due to low synthesis yields at higher lengths. This approach also requires clean rooms and specialised equipment [82].

Laser ablation is another useful approach for micropatterning and can be considered a specialised case of photolithography. Here, a high-energy laser beam is directed on a surface through a patterned mask which leads to ablation of the surface underneath the transparent regions of the photomask. This method suffers from the same limitations as all photolithographic techniques, but is able to produce highly resolved patterns of controlled depth at a fast rate, thus, being applicable to the production of patterned surface topographies as well as patterned surface chemistries. Thissen, et al. utilised laser ablation with the development of a plasma polymer based system that was shown to spatially confine cell growth [33, 35]. A schematic of the approach is shown in Figure 1.3. An allylamine plasma polymer (ALAPP) with amine functionality was formed and aldehyde terminated PEG was grafted to the surface by reductive amination. Subsequent laser ablation produced micron resolution patterned PEG and ALAPP regions (Figure 1.3A and B). Cells were shown to be confined to the ALAPP region to micron resolution over a four day period using a bovine corneal epithelial cell line (BCEp) (Figure 1.3C) [33]. This system was expanded to include a human embryonic kidney cell (HEK) line [35], and has recently been shown to spatially control protein adsorption [84].

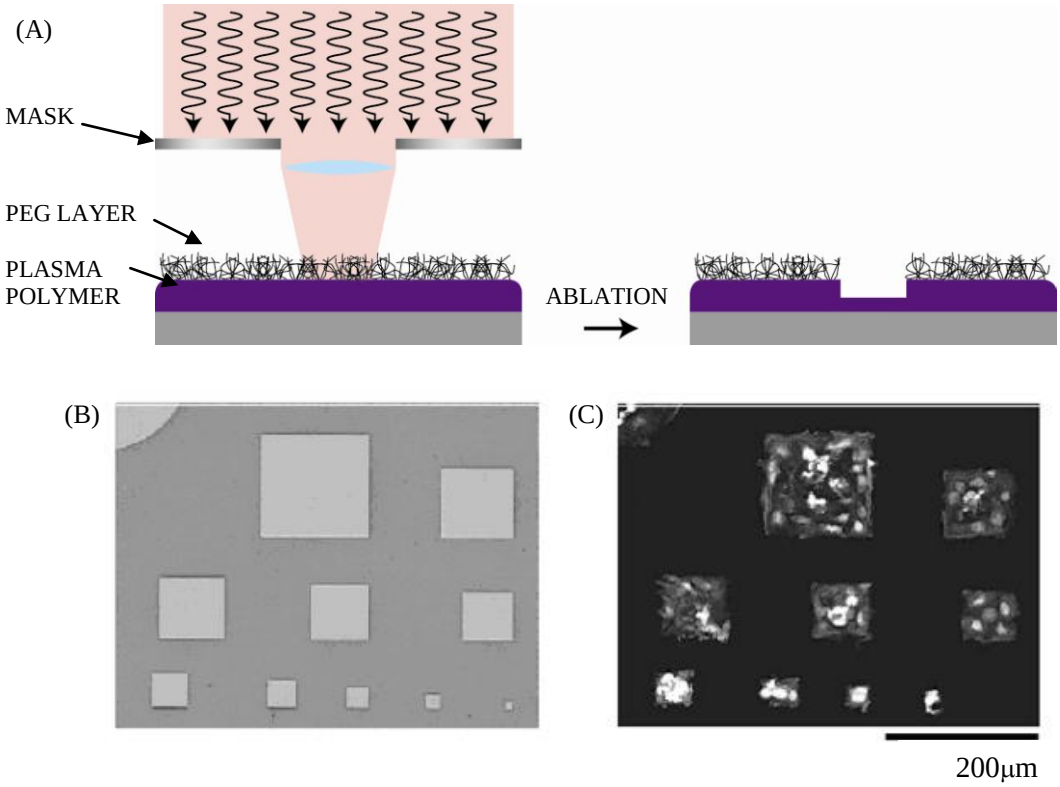

Figure 1.3. *(A) Schematic of two-dimensional patterning of the surface chemistry via excimer laser ablation. (B) Light microscopy image of laser ablated Si-ALAPP sample and (C) confocal scanning laser microscopy image of laser ablated Si–ALAPP–PEG sample after BCEp cell attachment and staining with PicoGreen®. From [33].*

1.2.2. Soft lithography

μCP is a soft lithography technique developed out of a need to pattern surfaces without the experimental difficulties and high costs of photolithography. Typically, this approach involves the once-off production of a mould, using photolithographic methods, that is patterned as the negative of the design of interest. This mould is then used to form a patterned stamp, typically composed of a crosslinked elastomeric polymer, like poly(dimethylsiloxane) (PDMS). The stamp is used to transfer a patterned image of an 'ink' molecule onto the desired substrate surface. The reusability of the photomask and low cost of the PDMS stamp make this technique an extremely cost effective and potentially high-throughput patterning alternative. Currently, this method is primarily used for the patterning of SAMs onto gold or silanes onto glass or silica surfaces. Thus, μCP suffers from a limited number of substrate surfaces and 'ink' molecules [85].

Zhang et al., [76] produced cell patterns using μCP of SAMs. A (11-mercaptoundec-1-yl)-hexa-(ethylene glycol) was printed onto gold coated silicon using a PDMS stamp with a topographical pattern. After SAM pattern transfer and washing, the surface was exposed to an engineered peptide strand containing an anchoring cysteine residue, an alanine linker and a Arg-Ala-Asp-Ser (RADS) cell adhesion motif linker group, which is based upon the recognition motif of ECM proteins and has been found to promote cell attachment [76]. This resulted in the spontaneous attachment of the peptide to the bare gold regions. Using this chemical pattern, cell patterns of micron resolution were formed using BAEc and human epidermal carcinoma cells. The use of normal mouse fibroblast (NIH3T3) cells,

however, demonstrated the ability of this cell type to grow over the ethylene glycol regions.

Hyun et al., [86] attempted to broaden the scope of μCP by developing surface chemistries that covalently react with the printed molecules. This was achieved by initially functionalising the surface of carboxylic acid functional polymers. The carboxylic acid groups were activated by reaction with pentafluorophenol to form pentafluorophenyl esters. Stamping of an amine functionaised molecule with biotin functionality resulted in its covalent attachment; thus, a patterned surface of biotin groups was produced. Subsequent addition of streptavidin modified molecules enabled the patterning of these molecules at the surface with micron resolution [86]. Tween 20 or BSA were adsorbed to the surface in order to saturate the surface with protein thereby minimising non-specific binding in between the printed regions. A similar technique was also used to initiate free radical polymerisation of PS by modifying a gold plated surface with thiols with terminal carboxylic acid functionality, subsequently converted to a pentafluorophenyl ester, and then printing a radical polymerisation initiator with a terminal amine. The initiator attached covalently to the SAM by formation of an amide bond. This enabled the spatially confined free radical polymerisation of PS at the surface upon addition of the polymerisation solution [87]. Here, cells preferentially attached to the SAM rather than the surface grafted PS layer.

In order to pattern cells upon a biodegradable surface, Kumar et al., [88] patterned a biocompatible chitosan substrate by μCP of random copolymers of methacrylic acid (MAA) and oligo(ethylene glycol) methacrylate (OEGMA). This polymer not only shows low fouling characteristics but, being an anionic polyelectrolyte, also binds through multivalent electrostatic interactions to the positively charged chitosan

substrate. A copolymer ratio for OEGMA/MA of 0.8 was found to be optimal composition for both ensuring strong polymer adsorption and cell resistance. Spatially controlled cell attachment with micron resolution was demonstrated using human microvascular endothelial cells.

1.2.3. Microfluidics

Microfluidics is a different approach to surface patterning whereupon biomolecule manipulation and spatial control is achieved by limiting the surface of the substrate that is accessible to the solvent carrying the biomolecule of interest. Microfluidic systems can be complicated, however, for a simple patterning experiment a patterned PDMS stamp, formed in a similar fashion to typical μCP experiments, has been shown to be very useful. The PDMS stamp is typically topographically patterned with grooves that form sealed channels when the stamp is pressed onto a hard, flat substrate surface.

Microfluidics often represents a cheaper and simpler solution than other patterning techniques and has the unique advantage of being able to separate and contain the reaction solution, enabling different solutions to be exposed to different locations on a particular surface. A further advantage is the gentler processing conditions in comparison to lithographical methods. This enables patterning over pre-attached cells or over proteins, which would be destroyed by harsher treatment conditions. However, there are limited number of channel geometries available and the resolution limit is generally lower than for lithographical approaches.

A multi-phenotype cell array was formed by encapsulating living cells within a hydrogel matrix, formed inside of a microfluidic channel made of PDMS [52]. Various cell lines in a hydrogel precursor solution were injected into parallel

channels flowing over a substrate surface. Cells were then trapped by irradiation of the channels with UV light through a photomask which resulted in cross-linking of the hydrogel. Subsequent removal of the microfluidic assembly left a patterned array of a multi-phenotype cell array. This system was able to sustain cell separation of murine fibroblast, murine hepatocytes and murine macrophages, keeping different cell types apart down to micron-size separation distances.

Patel et al., [74] produced a patterned culture of BAEc and pheochromocytoma (PC12) using microfluidics. A PDMS mould was formed containing grooves which formed microchannels when put in contact with a substrate. This enabled the spatial control of solvent flow over the surface. The substrate used was a film of PLA-PEG block copolymer modified with biotin. Flow of avidin over the film through the microchannels produced spatially activated regions on the substrate. Biotinylated peptides containing the RGD peptide or a laminin fragment were subsequently flowed through the microchannels to produce a surface conducive to cell attachment. Removal of the PDMS mould and seeding of the cells on the activated surface resulted in preferential attachment of the cells to the modified regions.

Takayama et al., [75] developed a method of cell patterning using combined laminar flows through capillary networks. In one experiment *Escherichia coli* (*E-coli*) was patterned to a surface by prepatterning a surface with a mannose containing protein (Figure 1.4A). Mannose was chosen because the cell membrane of *E-coli* is decorated with mannose-binding proteins. Subsequent incubation of *E-coli* with the patterned surface caused the adsorption of *E-coli* to the mannose-coated regions (Figure 1.4B). This technique also enabled the surface patterning of eukaryotic cells and proteins.

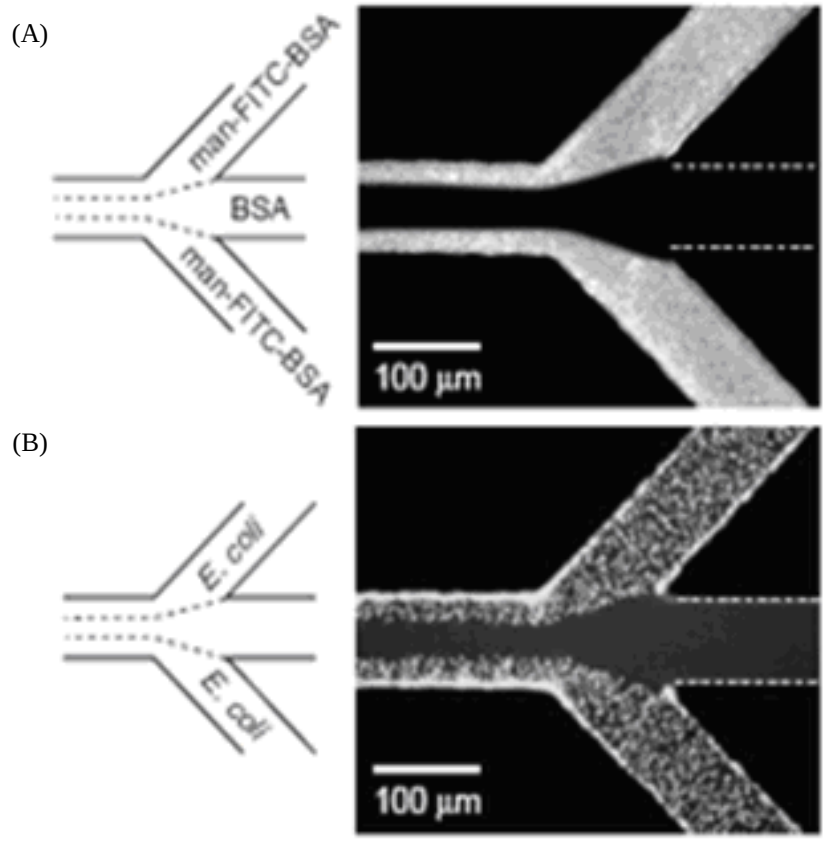

Figure 1.4. *Schematic representation of a laminar flow patterning experiment. Flow is from right to left. (A) Patterns of adsorbed protein created by laminar flow. Solutions of α-D-mannopyranosyl phenylisothiocyante,fluorescein isothiocyanate (FITC)–BSA (0.5 mg/ml in PBS) and BSA (10 mg/ml in PBS) were allowed to flow from the designated inlets into the main channel for 15 min under gentle aspiration, and the system was washed for 3 min with PBS. (B) The channels shown in (A) were filled with a suspension of E-coli RB 128 and allowed to stand for 10 min to allow adhesion; nonadherent cells were removed by washing with PBS. Cells were visualised with a fluorescent nucleic acid stain (Syto 9, 15 mM in PBS). Both micrographs were taken from the top of the capillary network looking through the PDMS. White dotted lines identify channels not visible with fluorescence microscopy. From [75].*

Finally, the Whitesides group has extensively investigated methods of cell patterning including μCP, microfluidics and laminar flow patterning. Much of this research has been previously reviewed [4, 85, 89-91].

1.2.4. Microelectronics

Microelectronics is a new field that exploits microcircuitry to manipulate biomolecules and cells. This technique has been developed by Huang et al., [77] to separate monocytic white blood cells and human T cells transformed with the oncogene Tax from human peripheral blood mononuclear cells as well as neuroblastoma cells from glioma cells on the basis of the distinct dielectric properties of the different cell types. Using a microelectronic chip array, effective separation and sorting of different cell types was demonstrated (Figure 1.5) by stepwise addition of cells (Figure 1.5A), separation by dielectrophoresis (Figure 1.5B), and then washing by buffer (Figure 1.5C and D). Microelectronic chips have also been shown to effectively control DNA adsorption and surface diffusion [92, 93]. This method is limited by the electrode pattern that can be fabricated. However, this approach does combine spatial control with switchability. Furthermore, as well as having the ability to pattern cells, it has the unique capability to control surface diffusion of cells, which adds a new dimension to advanced cellular manipulation.

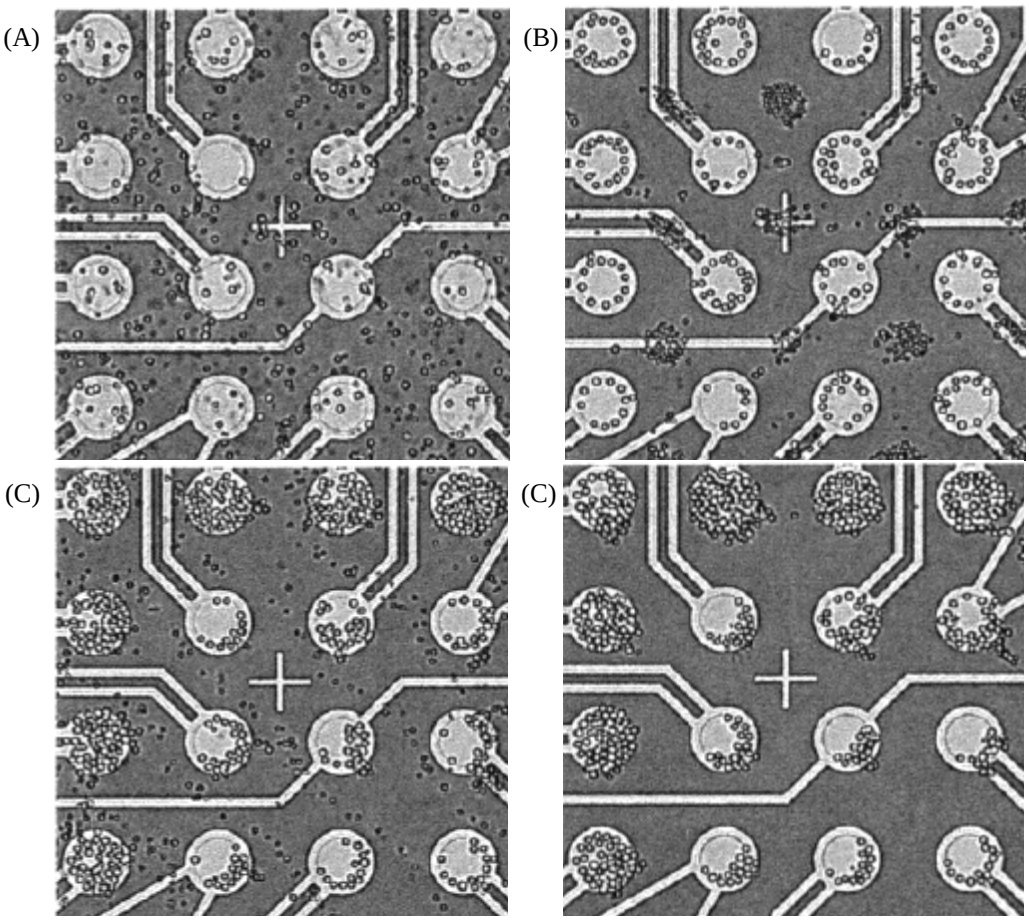

Figure 1.5. *The procedure of dielectrophoretic separation for a monocytic cell and peripheral blood mononuclear cell mixture. (A) The mixture is introduced to the array. (B) Monocytic cells are separated from peripheral blood mononuclear cells on array by dielectrophoresis 5 min after an ac voltage of 500 kHz frequency and 7 V amplitude is applied. Monocytic cells are collected on the electrodes and peripheral blood mononuclear cells are accumulated at the space between the electrodes. (C) Buffer is introduced from a reservoir to the array by fluid flow of 40 μL/min while the voltage is kept on. Peripheral blood mononuclear cells are carried away with the fluid stream. (D) Peripheral blood mononuclear cells are washed off from the array and monocytic cells are retained on the electrodes after 10 min of washing. From [77].*

1.2.5. Robotic contact and non-contact microprinting

Formation of micron resolution arrays is routinely achieved by robotic contact and non-contact microprinting. Robotic contact printing is achieved using a robotic spotter that first dips a pin with microscale diameter into a desired solution then spots the sample onto the substrate surface at a specified location. Two types of pins are typically used. First, a solid pin, which is commonly used for transferring proteins and other sticky molecules because of their ease of cleaning and second, a quilled pin that has a hollow centre that is able to draw up the solution and act as a reservoir allowing repeated spotting without re-dipping. Although, this increases the rate of microarray formation, these pins are much more difficult to keep clean and are, therefore, only suitable for 'non-sticky' molecules. Robotic non-contact printing is achieved by ejecting nano-litre volumes of the desired solution from a microcapillary onto specified positions on a surface. The advantage of this strategy is that common problems with pins, such as the risk of contamination if the pins are insufficiently cleaned, inhomogeneous spot geometry and variations in the dispensed volume, can be avoided [94], however, this approach typically suffers from 'splattering' of ejected volumes. Such strategies can be used to form DNA and protein microarrays and more recently also cell microarrays [8, 15, 82, 94-99].

This form of patterning is very effective at quickly and reproducibly producing micron resolution patterns that can be used for the cost effective, high-throughput analysis of proteins and DNA products using very little reagents on, typically, a single microscope slide. Microarrays were key to the success of the human genome project and will underpin further genomic analysis. The development of cell microarrays will further advance genomic analysis, and as the investigation of more

complex genomic questions proceeds, advanced surface chemistries for microarrays are required.

For example, one of the limitations with contact and non-contact printing is that although patterned arrays of DNA, proteins and other molecules are easy to form, there are very limited procedures to prevent cross-contamination or surface migration of arrayed species. One such strategy was developed by Yamauchi et al., [100], who spatially confined droplets of DNA solution at the surface by using a patterned SAM with regions of varied hydrophilicity produced using alkanethiols with different end-groups. Spots containing amine, hydroxyl and carboxylic acid groups separated by methyl terminated alkanethiols were shown to readily confine DNA containing water droplets. Baghdoyan et al., [101] sought to confine DNA by adding gelatin to the DNA mixture spotted onto a glass slide in the form of a microarray. This microarray was subsequently used for reverse transfection with the highest transfection efficiency occuring at 0.25-0.5% gelatin concentration. Addition of the gelatin was hoped to increase spatial control of DNA, however, this was not clearly demonstrated.

Spotted material can also be held in place by introducing various attractive interactions between the surface and the spotted material. For example, DNA is typically immobilised to a surface by either covalent interactions, such as immobilisation of thiolated ssDNA to gold surfaces, or non-covalent interactions such as adsorption of DNA to amine functionalised surfaces where electrostatic interactions dominate or interactions of biotin labelled ssDNA with avidin-presenting surfaces.

1.3. Surfaces with switchable properties

The development of switchable surfaces is a key enabling advancement for biodevice applications, including biomaterials and in particular tissue engineering and cell microarrays. Switchability, in essence, enables temporal control, adding another dimension to controlled biomolecular manipulation. These types of surfaces have already been instrumental in gaining a better understanding of biomolecular surfaces at the solid-liquid interface. Typically, DNA adsorption and desorption is temporally controlled by electrochemistry due to the polycationic nature of this biomolecule. On the other hand, a number of strategies to reversibly control cell and protein adhesion have been investigated and typically involve the use of hydrogels and switching of these polymers from a hydrophobic to hydrophilic state by temperature shifts about the lower critical solution temperature (LCST). Other triggers such as pH, specific ligand-receptor interactions and light have been investigated to evoke changes in hydrogel properties, including optical and density changes [70, 102-104].

1.3.1. Switchable DNA adsorption and desorption

Of great interest in terms of DNA surface manipulation are surfaces that can switch between a positive and negative surface charge, instigating temporal control over DNA adsorption and desorption. This is particularly important for TCMs whereupon adsorbed DNA must be released in order to be internalised by cells [1]. The ability to electro-stimulate the desorption of DNA has been studied extensively on gold [18, 105-108]. Wang et al., [105] demonstrated small amounts of DNA were released at voltages as low as -0.2 V, however, maximised DNA release was observed at -1.2 V. Jiang et al., [109] demonstrated by AFM analysis that the surface

morphology of adsorbed dsDNA could be changed by application of an increased magnitude of positive voltage.

Heller et al., [92] utilised the electro-responsive nature of DNA to form switchable checkerboard patterns of fluorescently labelled DNA on a microelectronic chip (Figure 1.6). By application of the appropriate positive and negative voltages DNA became spatially confined and a pattern was formed (Figure 1.6B). The inverse checkerboard pattern was formed by reversing the polarity of the applied voltage, and this reorientation was complete after 7 s (Figure 1.6D). Analysis of the stringency of DNA hybridisation was also achieved by controlling the surface diffusion of DNA oligonucleotides to move, on demand, over different regions functionalised with complementary or slightly mismatched oligonucleotides. Gilles et al., [93] used this technology to study single point mutations along the encoding a human mannose binding protein. Point mutations on genes are stable and can contribute to genetic disorders. Detection of a single point mutation is difficult considering the vast number of base pairs within any given genome. Gilles et al., [93] immobilised a number of different single stranded 123 bp oligonucleotide fragments of the allele of a mannose binding protein gene that differed by single point mutations at different sites on a 5 x 5 microelectronic chip array by streptavidin-biotin interactions. Application of a positive voltage at a particular site directed the immobilisation of each strand to a desired location. 21 or 22 bp oligonucleotide probes specific for the wild type allele or specific for a particular single point mutation were injected into the system and allowed to hybridise with immobilised DNA strands. By application of a negative voltage at a specific site the mismatched probe DNA could be driven away, whereas complementary probes would stay

behind. This strategy enabled the differentiation of the wild type and single point mutation oligonucleotides.

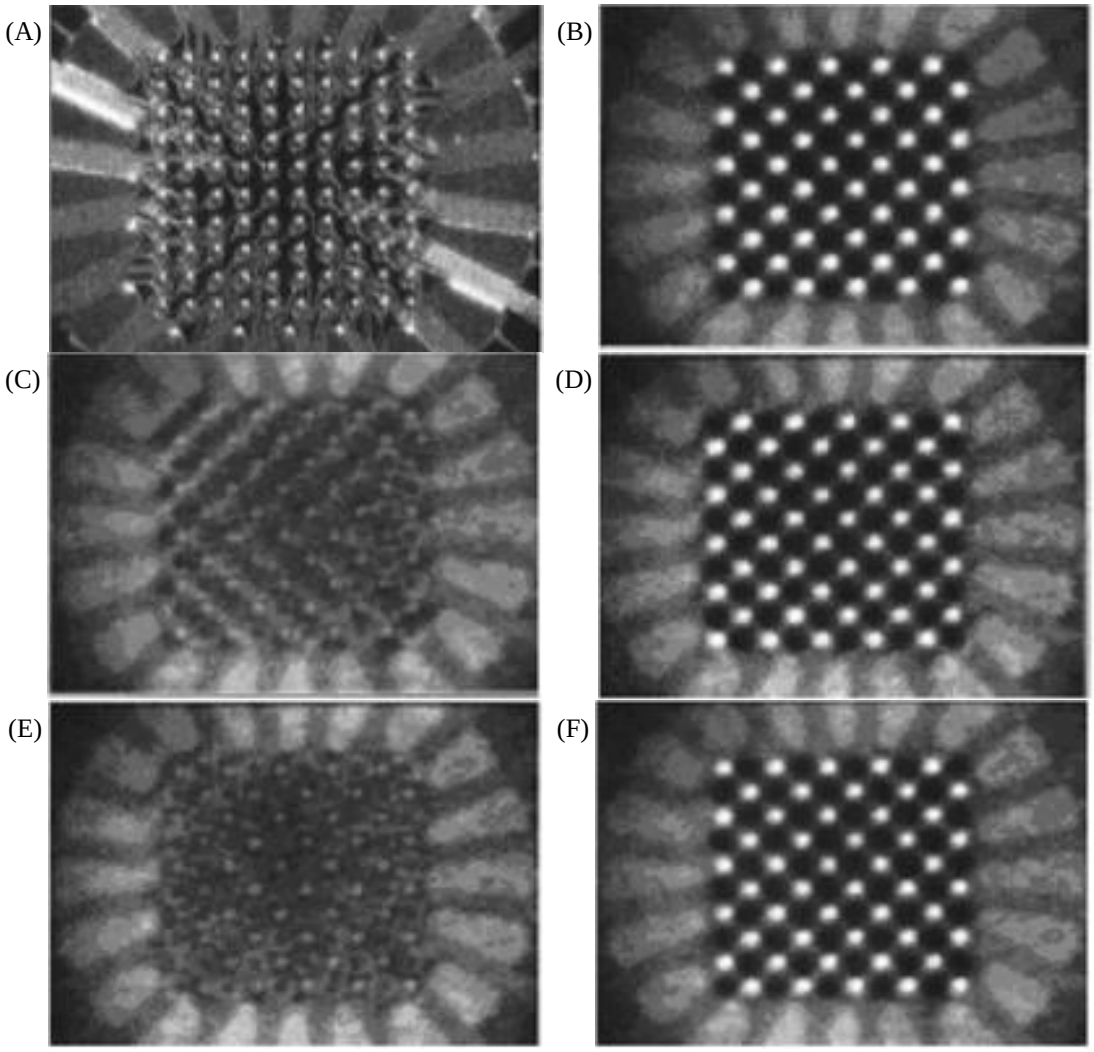

Figure 1.6. *DNA checkerboarding on 100 test site microelectronic array. In this experiment, all the microelectrodes on an agarose permlayer-coated 100 test site chip are biased positive or negative in a checkerboard pattern, with the polarity being reversed every 7 s. White light and fluorescent images are acquired in real-time using a charge coupled detector (CCD) camera and digital tape recorder. (A) 100 test site chip under white light and the 100 test site array with its 80 μm diameter microlocations. (B) Fluorescent image of the rapid accumulation of fluorescent probe at the positively (+) biased microlocation (white spots), and repulsion of the DNA probes from the negatively biased microlocation (black spots). Note that the fluorescent DNA over the unactivated counter-electrodes on the perimeter of the device is not influenced during the checkerboard experiment. (C) Fluorescent DNA in rapid transport as the polarity is reversed after 7 s. (D) Fluorescent probes now accumulating on the newly biased negative microlocations. (E) Transport after polarity reversal. (F) Accumulation of the fluorescent probes on the newly biased positive microlocations. From [92].*

1.3.2. Switchable surfaces for the control of proteins and cells

Controlling the behaviour of cells firstly requires the ability to manipulate the proteins that cells use to mediate their attachment to surfaces. Since a major driving force for protein adsorption is the surface dehydration associated with hydrophobic interactions, a focus for switching protein and cell attachment has been the production of surfaces that are able to alter their wettability when stimulated appropriately.

Okano's research group has developed a switchable polymeric surface for cell attachment using poly(*N*-isopropylacrylamide) (P*N*IPAAm), which switches from a hydrophilic state to a hydrophobic state by increasing the temperature above its LCST of 32 °C. Mammalian cells grew on the hydrophobic polymer, but not the hydrophilic polymer enabling cell adhesion to be turned on and off [110]. A pattern of the P*N*IPAAm was formed on a surface by UV-initiated polymerisation of the polymer through a metal photomask (Figure 1.7A) in direct contact with the surface such that P*N*IPAAm was only formed where the UV was not blocked. Using this technique, Yamato et al., [73] were able to produce an array of 1 mm diameter circular domains of P*N*IPAAm on a background of tissue culture grade PS. Seeded rat hepatocytes grew on both the P*N*IPAAm and the PS (Figure 1.7B), however, upon lowering the temperature, the cells detached from the P*N*IPAAm regions (Figure 1.7C). Upon raising the temperature to 37 °C seeded endothelial cells then attached to the P*N*IPAAm, generating a patterned cell co-culture (Figure 1.7D). Alternatively, Yamato et al., [64] seeded rat hepatocytes on the patterned P*N*IPAAm at 20 °C, below the LCST, such that cells only attached to the unmodified tissue culture grade PS. Subsequently seeded human fetal lung fibroblasts at 37 °C, above

the LCST, adhered to the P*N*IPAAm regions resulting in the formation of a heterotypic cell pattern. This approach has the advantage that cell detachment is not required. These methods are in principle adaptable to different cell types; theoretically any patterned co-culture of two cell types could be realised.

Extending the above approach, Edahiro et al., [104] developed a photoresponsive, switchable surface for cell attachment. The surface was composed of P*N*IPAAm with a photoresponsive chromophore that underwent isomerisation upon exposure to UV, and reversed upon exposure to light in the visible spectrum. A Chinese hamster ovary cell line (CHO-K1) was shown to have preferential attachment to the isomerised chromophore once exposed to UV, although the mechanism for this was not understood. This surface was patterned by irradiation of UV through a photomask to produce regions that cells preferentially attached to. Use of P*N*IPAAm enabled the bulk regeneration of the surface by reducing the temperature below P*N*IPAAm's LCST, causing the cells to detach.

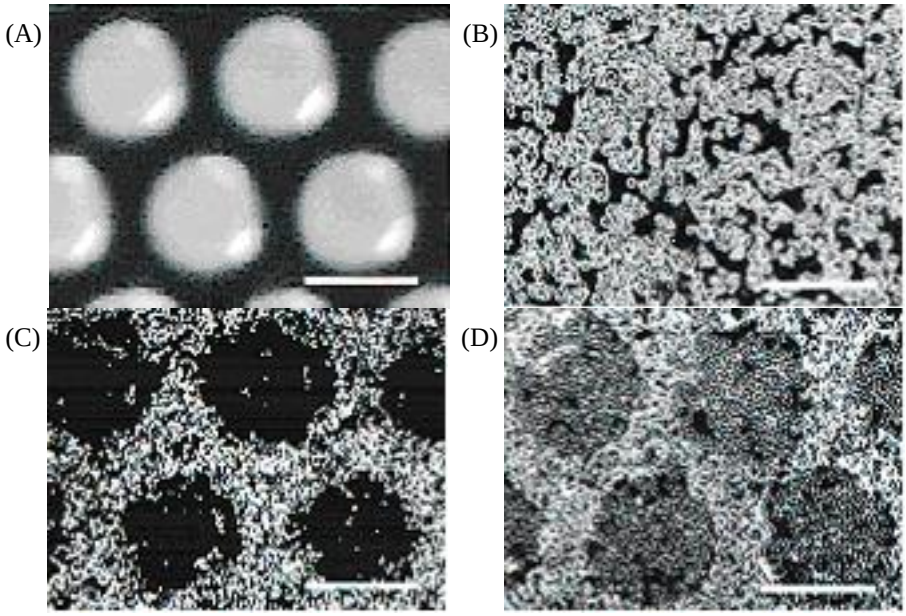

Figure 1.7. *A patterned co-culture of hepatocytes and endothelial calls. (A) The patterned metal mask with holes (1-mm diameter) used for surface patterning of PNIPAAm and PS. (B) Hepatocytes were seeded and adhered homogeneously on the whole surfaces at 37 °C. (C) By reducing the temperature, hepatocytes detached spontaneously and selectively from PNIPAAm -grafted domains. (D) Endothelial cells were seeded and cocultured with formerly seeded hepatocytes at 37 °C, forming a co-culture. Scale bars = (A, C, D) 1 mm and (B) 200 μm. From [73].*

Hyun et al., [111] developed a strategy for the switchable attachment of peptides modified with an elastin-like polypeptide moiety based upon the pentapeptide sequence of Val-Pro-Gly-X-Gly, where X is any amino acid other than proline. First, a patterned SAM with carboxylic acid functionality was formed by dip-pen nanolithography (DPN). An elastin-like polypeptide was then immobilised on the surface by 1-ethyl-3-(diethylamino)propyl carbodiimide (EDC) – *N*-hydroxysuccinimide (NHS) coupling. The elastin-like polypeptide is able to switch between a soluble and insoluble state at its LCST, which is tunable within the temperature range of 0-100 °C [111]. The LCST of the polypeptide could be reduced by increasing the ionic strength. Proteins could be adsorbed onto this surface in the collapsed state and released by reducing the temperature or the ionic strength.

Miyata et al., [103] developed a smart hydrogel that swelled 10% in the presence of an antigen. The creation of an antigen responsive hydrogel adds an important and more specific stimulus ligand-receptor interactions, to the toolbox for switchable surfaces. This hydrogel was formed by copolymerisation of acrylate derivatives of antigen and its corresponding antibody with acrylamide (AAm), such that the antibody-antigen interaction produced extensive crosslinking of the formed hydrogel. Addition of free antigen resulted in competitive binding of the free and immobilised antigen for the immobilised antibody, resulting in the breakdown of some of the hydrogel crosslinks and a concurrent swelling of the polymer. Miyata et al., [103] used a rabbit immunoglobulin (IgG) antibody as the antigen and a goat anti-rabbit IgG antibody. The swelling was reversible, antigen specific and occurred within a time scale of approximately 1 hr. This switchable swelling may be useful for certain cell culture applications, but it is of great interest for drug delivery on demand.

1.3.3. Alternative switchable surfaces

Switchability is not only limited to hydrogel systems. There is no lack of other, alternative strategies for the development of switchable surfaces with biodevice applications.

Lahann et al., [112] developed a surface coating on gold that transduced conformational changes in a low density SAM initiated by a voltage bias into wettability changes. The SAM was formed by attaching a large, cleavable head-group onto the alkanethiol used (16-mercapto)hexanoic acid (MHA), which limited the packing density of the SAM. After cleavage of the head group, a low density SAM remained. By application of a voltage bias, the ionised carboxylic acid group was attracted to the surface, producing a conformational change in the MHA backbone that exposed a hydrophobic loop at the solid liquid/interface, producing a reversible change in hydrophilicity, which was observed as a change in contact angle. Development of these self assembled surfaces offers exciting opportunities when applied to the manipulation of biomolecules.

Gillies et al., [113] developed a one-off switchable system using pH sensitive micelles. Linear-dendritic block copolymers containing hydrophobic head groups and PEG tails were formed, which spontaneously formed micelles in aqueous conditions. The hydrophobic head group contained a cyclic acetal linker that, upon hydrolysis at lower pH, decomposed, releasing trimethoxy benzene and producing a diol end group. This reaction increased the hydrophilicity of the head group and essentially removed the driving force for micelle formation, which led to their breakdown. The micelles were stable at physiological pH, however, lowering the pH to 5 caused the hydrolysis of the acetal group, initiating the destabilisation of the

micelles. This pH switch was used to deliver a hydrophobic dye encapsulated within the micelles on demand.

Ionov et al., [114] developed a mixed polyelectrolyte brush consisting of poly(acrylic acid) (PAA) and poly(2-vinyl pyridine) (PVPI) chains with pKa values of 6.7 and 3.2, respectively, whereupon PAA is negatively charged above pH=6.7 and PVPI is positively charged below pH=3.2. Switching of the pH from 3 to 9 caused a switching of the surface chemistry from a positive surface charge to negative. This change also instigated a rearrangement of the surface bound polymer, whereupon, at low pH PVPI was extended and, thus, dominated the surface character, whilst PAA was collapsed, hidden beneath the PVPI layer. At high pH this was reversed, with the PVPI collapsing and PAA extending. Concurrently, changing the pH altered the contact angle of the mixed polymer brush surface, whereupon the surface exhibited hydrophilic properties at high and low pH, but was more hydrophobic over the pH range of 4-8, where the surface had a near neutral surface charge.

Winkelmann et al., [115] produced a patterned surface with conductive and non-conductive regions of titanium and silicon, respectively by photolithographic microfabrication using a patterned photoresist. Surfaces with regions of different metals were also formed using this technique, enabling the study of protein and cell adhesion to a variety of metal interfaces [116]. Using this substrate, the controlled removal of adsorbed PLL-g-PEG at conductive regions was demonstrated by application of +1800 mV voltage bias, which enables further adsorption of functional molecules, in the present case human fibrinogen was adsorbed, to the re-exposed titanium layer [117].

1.4. Application of patterned and switchable surfaces – Microarrays

Recent years have seen the development of many advanced biomedical devices that have been useful for the study, advanced manipulation and application of biomolecules. Such devices have proved to be valuable tools for solving many biologically based problems, particularly in the field of medicine. Examples include microarrays, advanced drug delivery systems, biosensors and scaffolds for tissue engineering [3, 4, 6, 8]. Advanced manipulation of biomolecules requires surfaces or materials with the ability to adsorb, desorb, bind or prevent adsorption of biomolecules in localised regions combined with the ability to switch between these processes on demand or upon activation by a defined stimulus. A number of studies have been conducted in this field, with the main interest being in high-throughput DNA or protein manipulation and concurrently in controlling cell adhesion on microarray substrates. [15, 17, 66, 99]. With the completion of the human genome project has come the challenge of elucidating the function of the vast amount of genomic information within any single person's genotype. This has led to the emergence of three primary types of genomic analysis tools: protein microarrays, DNA microarrays and cell microarrays. The key advantage of microarray technology is the ability to conduct high-throughput studies with small amounts of analyte, enabling the rapid, inexpensive examination of genomics, proteomics or gene expression [118]. Cell microarrays offer an additional advantage in their ability to analyse the expression of genes and the function of proteins in a living cell where all the machinery is present to ensure correct function enabling the high-throughput validation of tens of thousands of gene and protein targets [119].

1.4.1. DNA microarrays

Vast amounts of research have been conducted with DNA microarrays and have proven to be of paramount importance in the areas of cancer research and other genetic based diseases. In fact, DNA microarray based research is an integral part of many physiological based research and has revolutionised genomic studies, which pertains not only to oncology but also neurology, pathology, psychology, pharmacology, pharmacogenomics and toxicogenomics, to list a few.

There are three methodologies utilised for DNA microarray formation. The first method is contact printing, achieved by dipping an array of pins into defined sample solutions and then, by use of high precision robotics, bringing the pins in contact with a substrate, with the formation of spots as sample solution is transferred from the pin to the substrate (see section 1.2.5). DNA is typically immobilised to a surface by either covalent interactions, such as immobilisation of thiolated ssDNA to gold surfaces, or non-covalent interactions such as adsorption of DNA to amine functionaised surfaces or interactions of biotin labelled ssDNA with surface immobilised avidin. Various studies have sought to control the density of immobilised DNA in order to optimise DNA hybridisation whilst seeking to minimise non-specific DNA adsorption [120-122]. The surface energy of the substrate material is an important factor determining how the surface wets, spreads and dries. Although microarray formation is commonly done on glass, lack of reproducibility in spot formation has led to efforts attempting to produce varied surfaces with desirable surface energies that produce more reproducible spots [123]. One such method is the formation of a mixed SAM containing different ratios of alcohol and methyl terminated alkanethiols. SAMs are well known for their ability to produce regular, reproducible, defect free films [124]. Optimisation of surface energy

by tuning the alcohol:methyl ratio enabled the successful formation of spots of Cy5 dye in dimethylsufoxide (DMSO) with reproducible size and shape [123].

As an alternative to contact printing, non-contact printing has been utilised for injecting preformed DNA onto a surface at specific locations with high precision. This method is commonly achieved by utilising inkjet technology. The advantage of this strategy is that problems with pin transfer, such as risk of transfer of materials, variation in spot formation and the influence of metallic pins, can be avoided [94].

Furthermore, the development of an on chip DNA growth technique has also been successful achieved (see section 1.2.1).

DNA microarrays have previously been reviewed in detail [14-16, 82, 96, 125-130].

1.4.2. Protein microarrays

Protein microarrays are predominately used to investigate the abundance of specific proteins, usually achieved by the use of an antibody microarray, and how proteins interact with each other or with small molecules [3]. Protein microarrays are formed by adsorbing or covalently binding a number of different proteins in an array format. Washing with a labelled target molecule or a number of differently labelled targets, consisting of other proteins or small molecules, can result in the determination of the protein-protein or protein-small molecule interactions. Protein microarrays have been extended to include peptide microarrays, which, like protein arrays, are a series of small peptides immobilised onto a surface in an array format. Peptide arrays are advantageous over protein arrays in that peptides can be synthetically made and are, thus, easier to purify and enable the study of protein fragments, specifically the reactive sites [3].

A major problem with protein microarrays is the ability to attach the protein whist maintaining its functionality. Various methods of attachment are used including physioadsorption, metal complexation, covalent attachment, electrostatic attraction or by biological interaction (biotin-avidin) (see section 1.2.5) [79, 131, 132]. The common problem is that the attachment is either weak, enabling protein to be washed away or replaced with another protein, or the binding denatures or sterically hinders the binding site. One strategy to combat this was achieved by the production of a PLA-PEG film containing biotin [133]. Addition of avidin as a linker molecule enabled the potential attachment of biotin modified proteins, where if correctly modified, would result in correctly orientated proteins that are strongly attached. Another similar strategy is the use of His-proteins, which form a chelate complex with Ni^{2+}. Surfaces modified with carboxylic functionality are able to form such Ni^{2+} complexes and effectively immobilise His-proteins, which can be subsequently removed by addition of either EDTA that chelates Ni^{2+}, thus, breaking apart the complex formed with the his-protein, or imidiazole that competitively binds to the Ni^{2+} comples in place of the his-proteins. Such strategies are often used in protein purification. This and similar strategies involving protein modification and controlled attachment currently provide the means of effective protein microarray formation without compromising on protein activity.

Another problem associated with protein microarrays is storage, handling and purification of an array of proteins. Formed microarrays may have limited temperature ranges and often have short shelf lives. Ramachadran at al., [134] have developed a technique for the production of proteins *in situ* on the microarray on demand by utilising *in vitro* DNA transcription and translation. Complementary DNA (cDNA), which is DNA reverse transcribed from mRNA whereupon the RNA

has undergone post-transcriptional processing including gene shuffling and intron excision, is spotted onto the surface. cDNA libraries are usually constructed to include every gene within a genome and the genes are often inserted into plasmid DNA form. Once spotted and immobilised by a biotin-avidin interaction, use of cell-free *in vitro* transcription and translation machinery produces the encoded proteins *in situ* at addressed location. Addition of C-terminal glutathione *S*-transferase tag and surface-tethered glutathione *S*-transferase antibody enabled the immobilisation of the proteins *in situ*. Protein arrays produced in this fashion were used to perform typical protein microarray analysis, including protein recognition with antibodies and protein-protein interactions where the probe protein was also translated *in vitro*, but without a tag to prevent immobilisation [134].

Protein microarrays have previously been reviewed in depth and further information can be found in the following reviews [97-99, 135-142].

1.4.3. Polymer microarrays

Polymer microarrays are a recently developed microarray format that primarily allows for the screening of cell-material interactions and are a key enabling device for the development of new materials for specific biomaterial applications. Typically an array of polymer materials is formed on a low-fouling coating and subsequently exposed to cell culture conditions [143, 144]. Polymer spots inducing desirable behaviour to attached cells can be readily identified.

Anderson et al., [145] developed a method for the *in situ* polymerisation of polymer materials in an array format. Various combinations of acrylate monomers were deposited with a radical initiator in an array format on a low-fouling poly(hydroxyethyl methacrylate) coating. Upon UV irradiation rigid polymer spots

attached to the coating were formed that could be used for subsequent assay of cell-material interactions. Automated X-ray photoelectron spectroscopy (XPS), contact angle and time of flight secondary ion mass spectroscopy (ToF-SIMS) analysis allows for the rapid characterisation of the formed polymer array and the ability to match observed cellular behaviour with particular chemical or structural properties of individual polymer spots [146].

1.4.4. Cell microarrays

A number of different approaches to cell microarrays have been explored enabling the investigation of gene expression, cell surface interactions particularly with antibodies, ECM composition, cell migration and proliferation, the effects of drugs on cellular activity and a number of other areas [45]. A review of cell microarrays in general has been recently published [45]. Of great interest has been the development of TCMs [8]. A schematic representation of the TCM formation is shown in Figure 1.8. TCM formation consists of four key steps. Firstly, a nucleic acid microarray is formed using typical DNA microarray formation techniques, which have previously been reviewed in detail [15, 16, 82, 96]. Secondly, cells are seeded and attach onto the surface. Thirdly, DNA detaches from the surface and is taken up by the cells and finally overexpressed within the cells or, when using RNA interference (RNAi), the target gene is silenced. Various genomic studies have been undertaken utilising TCMs, such as the determination of the cellular position of expressed proteins, detection of gene products involved with apoptosis and the screening of agonists and antagonists of G-protein coupled receptors (GPCRs) [8, 147, 148]. A list of current applications of cell microarrays using transfection is shown in Table 1.1.

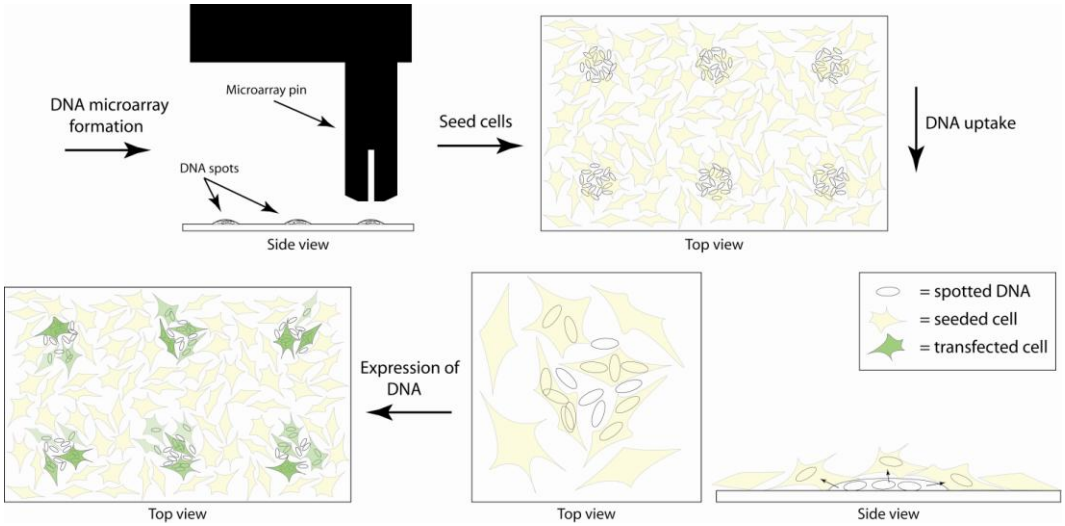

Figure 1.8. *Schematic of the formation of a transfected cell microarray. DNA is transferred to a solid surface using a robotic transfer device. Cells are then seeded and attach to the surface. Cells that attach to the regions where DNA was deposited will firstly uptake and then express the DNA [149]. Schematic not drawn to scale.*

Table 1.1. *Outline of the applications of transfected cell microarrays.*

Application	References
Screening of siRNAs and shRNAs for effective silencing of target genes by RNAi. Use of RNAi for gene function determination.	[150-154]
Development of a reporter system for monitoring the expression of proteins associated with signalling pathways.	[155, 156]
Assignment of the position of subcellular localised proteins.	[147, 157]
Automated analysis of nuclear area, cell area and number of cells.	[158]
Screening for agonists and antagonists of GPCRs.	[148]
Screening for gene products that are involved with kinase signalling pathways, stimulation of apoptosis or cell-cell adhesion.	[8]
Monitoring the interaction of receptor molecules with membrane-bound signal proteins.	[8, 159]
Use of linear polymerase chain reaction (PCR) products for TCM format.	[155]

Overexpressing a defined cDNA is useful for the genomic analysis of proteins of interest that are functioning within their natural environments. Receptor proteins have been a focus of these studies and, in particular, the screening of activators and inhibitors of GPCRs [148]. As GPCRs are currently the target of 40% of commercial drugs, determining the functionality and binding properties of unstudied GPCRs will likely determine other potential drug targets. Furthermore, microarray analysis of these receptors in a microarray format enables the high-throughput examination of the selectivity of drugs and identification of any side-reactions.

Recently, RNAi has been demonstrated on TCMs [150, 152-154]. RNAi for mammalian cells involves the use of either short (<30 nucleotides) interfering RNA molecules or the transfection of DNA constructs that induce the expression of short hairpin RNAs that interfere with the homologous messenger RNA to specifically silence the corresponding gene [160]. Double-stranded RNA strands longer than 30 nucleotides activate the interferon response within mammalian cells and are, therefore, not only useful for gene silencing assays in prokaryotic cells. Combined with TCMs, RNAi enables the high-throughput determination of the function of genes by observing the phenotypic effects of gene silencing. RNAi can be of even greater use for determining gene function than the over-expression of cDNAs, since the latter can give misleading results through the formation of unnatural phenotypes.

TCMs have three primary limitations. Firstly, an appropriately patterned substrate material that allows spatial control over both DNA and cells in order to effectively separate adjacent cell and DNA sites must be developed. This is important, not only for stringent confinement of DNA and cells to localised and addressable locations, but also for ease of analysis, whereupon, the lawn of non-transfected cells does not need to be discerned from transfected cells [45]. Secondly, low transfection

efficiencies are reported for reverse transfection experiments [29, 35, 101, 159]. Low transfection efficiencies are detrimental as each colony may consist of as little as 30 cells, thus, in order to gain statistically credible data close to 100% transfection efficiency is desirable. This is often not observed, limiting the technique to cell lines such as HEK and monkey kidney fibroblasts (COS) where relatively high transfection efficiencies are easily obtained [161], thus restricting the ability to study genes in the desired cell lines. Lastly, the requirement to accurately observe phenotypic changes within tens of thousands of cell colonies in a high-throughput fashion imposes a formidable challenge for TCM assay development.

1.4.4.1. Methods to generate DNA microarrays

The first step for TCM construction is the spatially controlled deposition of DNA in a microarray format. This is typically achieved by either contact printing or non-contact printing with a robotic spotter, μCP or on-chip DNA synthesis [15, 82, 94, 162]. DNA-gelatin mixtures are often utilised to ensure spatial confinement of DNA by physical entrapment [8, 101]. This can also be achieved by producing a surface with variations in hydrophilicity that effectively confine a DNA droplet to the hydrophilic regions, enabling DNA adsorption only in confined regions [100].

Spatial control of DNA has also been achieved utilising the electro-responsive nature of DNA to form switchable patterns of DNA on a microelectronics chip [92]. By application of the appropriate positive and negative voltages DNA can be spatially confined. The advantage of this system is the ability to induce surface diffusion of DNA by reversing the polarity of the applied voltage. This technique is limited by the pattern of the electrodes but combines spatial control with switchability for advanced DNA surface manipulation.

Although currently not utilised for TCM applications, the development of an on-chip DNA growth method enables high-density, spatially controlled DNA arrays to be formed that could be advantageous for TCMs provided this DNA could be taken up and successfully expressed by cells (see section 1.2.1).

For TCM applications obtaining high DNA surface concentrations is essential for enhancing transfection efficiencies. It is unlikely that DNA deposited at a particular location is taken up entirely by the cells seeded upon it, thus, one must ensure an excess of DNA by producing surface chemistries with the ability to sustain high DNA surface concentrations. However, DNA immobilisation must also be reversible so that the DNA can be subsequently taken up by cells [34]. Indeed, transfection efficiency has been shown to vary significantly with varying surface chemistries [29], demonstrating the importance of optimising DNA-surface interactions for TCM applications. In order to achieve this various surface chemistries and their interactions with DNA have been studied.

Generally, the adsorption of DNA onto a given surface is based upon two binding interactions, attractive electrostatic forces governed by the negative charge of the DNA phosphate groups and oppositely charged functional groups on the substrate surface as well as hydrophobic effects in aqueous medium associated with the interactions of nucleobases and nonpolar surface-bound moities (see section 1.1.2) [18, 19]. Hydrophobic effects are more significant for ssDNA as compared with dsDNA due to the role of nucleobases, which are imbedded within the core of a helical dsDNA strand but comparatively exposed within ssDNA.

Electrostatic interactions of surfaces with DNA have been the primary focus for enabling high DNA surface concentrations; thus, formation of positively charged surfaces is often desirable for DNA adsorption experiments. This is commonly

achieved by the production of amine rich surfaces, which are typically protonated at physiological pH. Common strategies for the formation of aminated surfaces are silanisation of glass, the adsorption of cationic molecules such as PEI and plasma polymerisation of amine containing monomers [24, 34, 42]. The use of cationic polymers is particularly useful for TCM applications as complexes formed between DNA and unbound cationic polymers can efficiently permeate the cellular membrane [163].

1.4.4.2. Cell seeding and attachment

Successful seeding and attachment of cells requires an adherent cell line and a biocompatible surface. There are many suitable surfaces for cell microarray formation including glass, silicon and tissue culture polystyrene, all of which are amenable to a variety of surface modifications. Furthermore, a method has been developed whereupon non-adherent cell lines are immobilised on a surface, increasing the scope of cell lines applicable for the TCM method [164]. This was achieved by modifying a glass surface with oleyl poly(ethylene glycol) ether, which acts as an anchor for subsequent membrane attachment.

TCMs can benefit from advanced cell patterning techniques if these methods can restrict cell attachment to regions where DNA has been deposited previously and prevent the migration of DNA or transfected cells. Research on cell patterning has focussed on producing ‘black-and-white’ patterned surfaces containing both bioactive regions that support protein and cell attachment, and non-adhesive regions that resist biomolecule attachment. As cells attach to a surface by the production of surface adhering proteins the manipulation of protein adsorption and cell attachment are closely related. A technical review of the various approaches to modifying surfaces for patterned cell growth has been recently published [66]. Strategies for

surface patterning are varied and include microfluidics, μCP, ultramicroelectronics, photolithography, soft lithography, laser ablation and robotic contact and non-contact printing (see section 1.2) [33-35, 52, 53, 74, 76-78, 80, 86, 88, 95, 165]. An outline of the various surface patterning techniques, their advantages and disadvantages and their application to TCMs is given in Table 1.2. Utilising methods including microfluidics and photolithography, the resolution of cell microarrays has been reduced to single-cell microarrays and these microarrays have been used for biological analysis such as monitoring Ca^{2+} mobilisation [165, 166].

A common strategy for promoting cell attachment is the functionalisation of a surface with proteins such as collagen, fibronectin, vitronectin or the immobilisation of integrin binding peptides such as RGD, a peptide representing a cell adhesion mediating sequence within fibronectin [33, 52-54].

Apart from effects based on the surface chemistry, the effects of topographical cues to initiate and control the attachment, proliferation, orientation and migration of cells and tissue samples on surfaces have been investigated (see section 1.1.3) [47, 56, 57, 62].

Table 1.2. *Description of various surface patterning techniques for spatially controlled cell attachment.*

Technique	Advantages	Disadvantages	Typical procedures	Cell microarray application	References
Photolithography	Sub-micron resolution pattern formation.	Rigorous laboratory procedures. Expensive maintenance costs. Clean environment required.	Deposition of a photoreist or a photoactive layer. Subsequently irradiate, typically with UV light, through a photomask.	Formation of patterned bioactive or non-fouling regions for spatially controlled cell attachment. Formation of topographical cues for directed cell attachment and outgrowth.	[53, 78, 80]
Laser ablation	Sub-micron resolution pattern formation. Controlled depth and rate of ablation. Adaptable to any ablatable surface.	Rigorous laboratory procedures. Expensive maintenance costs. Clean environment required.	Formation of an ablatable layer with subsequent laser irradiation, either thorough a mask or by a focussed layer.	Formation of patterned bioactive or non-fouling regions for spatially controlled cell attachment. Formation of topographical cues for directed cell attachment and outgrowth.	[33-35]
Microcontact printing	Lower costs and relative experimental ease. Micron resolution pattern formation.	Limited number of molecules and substratum that can be used.	Formation of a photomask for the formation of a patterned stamp. Stamp is then used to transfer thiolated SAMs onto gold or silanes onto glass.	Deposition of either bioactive or non-fouling molecules for spatially directed cell attachment	[76, 86, 88]
Microfluidics	Relative experimental ease. Ability to separate reaction solution.	Poor resolution. Limited patterns.	Formation of a PDMS mask from a mold contining grooves. Sealing of mask onto surface creates channels. Solution containing desired molecules or cells is flowed through channels.	Formation of single-cell microarrays and arrays of various cell lines by controlled delivery of cells to localised regions.	[52, 74, 165]
Microelectronics	Separates various cell types. Induces surface migration of cells. Effectively positions and holds cells spatially.	Poor resolution. Limited to the dimensions of the microelectronic s chip. Only applicable to limited cell lines.	Use of microelectronics chip containing an array of individual electrodes is used to apply specific voltages to localised regions.	Application of appropriate voltages leads to cell separation on the basis of distinct dielectric properties between different cell types.	[77]
Robotic contact and non-contact printing	Quick and simple pattern formation. Micron resolution.	No mechanism for spatial confinement once patterning completed. Patterned shape limited to pin (contact printing). Uneven adsorption of biomolecules in each spot.	Spotting of biomolecules onto localised addressable regions.	Cell microarrays have been formed spotting cell patterns by robotic arms.	[94, 95]

Several strategies have demonstrated the ability to reduce protein adsorption, including the surface immobilisation of carbohydrates. However, by far the most commonly used and most effective strategy for the formation of non-fouling surfaces available today is the surface grafting of PEG [66]. A number of surface modification methods designed to reduce non-specific protein adsorption based on PEG coatings have been discussed previously [66].

1.4.4.3. DNA uptake

The third step in the fabrication of a TCM is the uptake of DNA by cells from the surface. This step includes three primary processes, release of DNA from the surface, diffusion of DNA to the cell surface and transportation of DNA across the cellular membrane and, typically, into the nucleus. Low transfection efficiencies are likely due to a failure in one of these processes.

As previously discussed, DNA adsorbs to surfaces by hydrophobic or electrostatic interactions (see section 1.1.2). Therefore, releasing the DNA from the surface is achieved by reversing these interactions. Switching the hydrophobicity at a surface can be achieved using hydrogels that respond to temperature changes at their lower critical solution temperature by altering between hydrophobic and hydrophilic states. This system is readily used for producing switchable cell attachment [64, 73], but has not been demonstrated for DNA interactions. Hydrophobicity can also be altered by solvent changes, however, this is unsuitable for cell cultures. The easiest means of effecting DNA release from a surface is by changing the surface charge, typically achieved by application of a voltage. Temporal control of DNA adsorption and desorption has been extensively studied by electrochemical techniques [18, 106, 108].

Once released from the surface, DNA is able to diffuse in solution, soon contacting the surface of cells that are growing above the DNA loaded patches. From here, the polyanionic DNA must permeate the cell membrane, which typically also has an overall negative charge. From the methods usually used to facilitate transfection, only few can be used in a solid phase transfection scenario.

Commonly, transfection reagents such as the commercially available Effectene and Lipofectamine^{TM} reagents, are used to assist transfection in TCMs [8, 35, 101]. The transfection reagent is either mixed with DNA before spotting or afterwards, added on top of the DNA spots. Alternatively, inclusion of cationic polymers into the substratum enhances transfection due to the increased capacity for the surface binding of DNA resulting in an increased DNA concentration at the surface of attached cells and increased membrane permeability of DNA-cationic polymer complexes, which have a lower surface charge than bare DNA [163, 167].

A limited number of transfection methods have also been described for TCM applications as alternatives to the use of cationic polymers. Lentiviruses have been utilised for the transfection of a number of primary and transformed mammalian cells in a cell microarray format for both the overexpression of a particular gene and RNAi studies [168, 169]. Lentiviruses are advantageous over other transfection techniques in their ability to transfect a wide variety of cell types with a high efficiency. Another method developed to enhance DNA uptake is the use of electroporation [170]. The advantages of this approach is its ability to initiate both DNA release from the surface and subsequent uptake into the cells through transient pores in response to the application of a voltage.

Of particular interest have been studies showing that the interactions of ECM proteins enhance transfection efficiency [171-173]. The particular type of ECM

protein that will enhance transfection efficiency depends on the cell line used. For example, the use of collagen IV as a surface coating improved transfection efficiency up to 8-fold in PC12, where fibronectin showed little effect [172]. This contrasts with previous studies done with HEK cells, human mesenchymal stem cells, Henrietta Lack (HeLa) cells, normal mouse fibroblasts and human hepatocellular liver carcinoma where high solid state transfection efficiencies were achieved with the addition of fibronectin [173]. The postulated mechanism for ECM proteins promoting transfection efficiency is the mechanical stress that these proteins inflict upon the cellular membrane, easing the uptake of DNA through the stressed cell membrane.

A number of proteins such as transferrin, adenoviral penton protein and human immunodeficiency virus Tat protein have also been shown to improve transfection efficiency and have been applied to TCM applications successfully [155]. Of all proteins used, inclusion of the adenoviral penton protein has achieved the highest transfection efficiencies. The mechanism for this is unknown.

1.4.4.4. DNA expression

The final step in the formation of a TCM is the expression of the uptaken DNA by the cell. This process is complex and beyond the scope of this chapter.

Green fluorescing protein (GFP) is often used as a reporter protein to ensure that a particular plasmid has been taken up and is being expressed intracellularly. Interestingly, a variance in the intensity of fluorescence of cells transfected by a GFP encoding gene has been noted and the fluorescence intensity seems to correlate with the number of plasmids or DNA fragments taken up by a cell.

One of the challenges for TCMs is the analysis of potentially thousands of individual cell colonies each displaying a different phenotype, often requiring

imaging not only of cell clusters, but also of single cells and at the subcellular level. Fluorescent microarray scanners are useful for the quick production of an image of the entire array, however, the development of fluorescence scanning microscopes, which are able to automatically locate, focus and acquire images of individual cells, enables high-resolution, subcellular imaging. Considering the vast number of images potentially generated an automated, high-throughput, reproducible strategy for subsequent analysis is important. A number of software programs have been developed for this purpose. One such approach enabled survey analysis of nuclear area, total cell area and number of cells by software analysis of the red, blue and green channels from fluorescence images taken of cells stained with various subcellular localised dyes enabled the [158]. The automated accrual of images of transfected cells has also been used to generate an automated classification system whereupon. By reference to a set of assigned images, the subcellular position of expressed GFP tagged proteins was detected and assigned [147].

1.5. Conclusion and future perspectives

The development of mechanisms and devices for the advanced surface manipulation of biomolecules is an exciting field of research that promises valuable tools for scientists pursuing sophisticated biological studies or developing advanced biodevices. Surface manipulation of biomolecules currently permits spatial control over biomolecule placement via patterned surface chemistry or topography and temporal control over the attachment, adsorption or association and detachment, desorption or dissociation events via switchable surfaces. Limited research has also demonstrated control over surface diffusion of biomolecules, which is an exciting new dimension to the manipulation of biomolecules on a surface. A number of

techniques have been developed to achieve these various abilities, however, the most interesting and useful systems are those that combine patterns on the micro- or even nano-scale and switchable architectures to achieve both temporal and spatial control over biomolecule surface interactions.

Recent studies have shed some light on understanding the processes involved with the adsorption and desorption of DNA at the solid/liquid interface. As a stable polyelectrolyte with in-built molecular recognition properties, DNA is an interesting and unique biomolecule, and owing to its biological importance it is an ideal focus for the development of biodevices. The manipulation of DNA on a surface requires control over electrostatic and hydrophobic interactions, the driving force for DNA adsorption. Typically, low pH and high salt concentrations enhance DNA adsorption. These principles have been utilised recently to improve DNA based biodevices for example TCMs.

The manipulation of cells at surfaces has been an area of significant recent research interest. Chemical, biological and topographical cues have been all shown to be effective in controlling cell surface interactions. Generally, the control of cell attachment is achieved via the manipulation of the proteins that the cells themselves produce to mediate their attachment to surfaces. Protein adsorption, driven largely by hydrophobic forces, has also attracted much interest, and the ability to pattern surfaces with proteins enables cell patterning. Key discoveries have led to patterned cultures of cells with micron resolution using a wide variety of cell types and substrate materials. The development of switchable systems for cell attachment has enabled formation of patterned co-cultures of cells and opens up exciting possibilities for applications in tissue engineering and stem cell research.

A number of biodevice applications will benefit from the further development of biomolecules manipulation and an advanced understanding of the underlying phenomena. In particular, the recently developed transfected cell microarray method will be optimised and its scope expanded to a wide range of cell lines and primary cells. Already key steps have been taken to confine cell colonies to localised regions, to improve transfection efficiency, to adapt the approach to cells that are notoriously difficult to transfect or even non-adherent. One can easily envisage systems being developed where cells and DNA are delivered with high precision and speed for formation of high-density cell co-cultures that will not only be useful for genomic and proteomic analysis, but also for stem cell differentiation experiments and tissue engineering.

The development of TCMs is both an exciting and important development for the high-throughput determination of gene function, which is important for combating genetic based disorders such as cancer and for the identification of potential drug pathways. The increased understanding of DNA-surface interactions and the development of advanced material surfaces with the ability to temporally and spatially control biomolecule manipulation, formed by the use of high-resolution patterning techniques, provides the means to develop highly functional and reliable platforms for advanced genomic analysis. The continued development of RNAi on TCMs for high-throughput loss-of-function studies and the development of methods enabling highly resolved subcellular phenotypic examination will enable more in-depth studies, not only into gene function but also into the machinery involved in gene expression. The means to optimise the DNA microarray formation, cell seeding, DNA uptake and expression are within grasp and no doubt will result in the implementation of an advanced genomic analysis tool that is adaptable to a wide

variety of gene functions and cell lines. Furthermore, the development of cDNA and RNAi genome wide libraries would enable the high-throughput, rapid and inexpensive analysis of entire genomes, with each gene either overexpressed or silenced at defined cell clusters, all upon a single glass slide.

However, a number of key issues must be resolved before TCMs can become the high-throughput tool that is desired. Methods to manage low transfection efficiencies and effective colony separation needs to be developed. Recent developments discussed in this chapter point to a solution of these problems. The greatest foreseeable challenge is the ability to process tens of thousands of colonies, all potentially with a varied phenotype. A number of automated fluorescence systems are being developed, and further progress in this area is essential for the successful implementation of TCMs. The use of fluorescence tags and GFP as a reporter gene are pivotal to this analysis and have been used successfully for a number of gene function studies. However, the further development of other screening methods that infer gene function would broaden the scope of TCM applications to study phenotypes where fluorescence tagging or staining is not viable.