

Chapter 23

Using Self-Assembled Monolayers That Present Oligo(ethylene glycol) Groups To Control the Interactions of Proteins with Surfaces

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This chapter reviews the use of self-assembled monolayers of alkanethiolates on gold to control the interactions of proteins and cells with man-made materials. The work is based on the ability of monolayers that present oligo(ethylene glycol) groups to resist the non-specific adsorption of protein. The chapter describes the use of functionalized monolayers for the bio- and chemo-specific adsorption of proteins. The chapter concludes with a discussion of techniques that can pattern the formation of monolayers and that can prepare tailored substrates for the control of cell attachment.

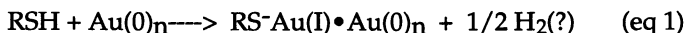
The property of poly(ethylene glycol) (PEG) to resist the non-specific adsorption of protein has made this material the standard choice for applications requiring inert surfaces. There exist a variety of excellent strategies for tailoring the surfaces of materials with PEG. Most of the methods of using PEG are empirical, and a mechanistic understanding of the ability of PEG to resist adsorption is still incomplete. As a consequence, the structural parameters of PEG that make surfaces presenting it unable to adsorb proteins (that is, inert to adsorption) are still not well understood, and it is not yet routine to design new inert materials—or even simple derivatives of PEG—from basic principles.

We have used self-assembled monolayers (SAMs) of alkanethiolates on gold that present oligomers of the ethylene glycol group ($-\text{EG}_n\text{OH}$, $n = 2-6$, and $-\text{EG}_6\text{OCH}_3$) as model surfaces with which to study the properties of materials tailored with PEG (1-4). Several considerations make this class of SAMs the best that is currently available for fundamental studies of the relationships between the structure of a material and its interfacial properties. The structure of these interfaces is reasonably well-defined and stable over the intervals required for experiments involving protein adsorption and cell attachment. They can be systematically tailored using routine organic synthetic methods (4-6). Additional considerations that make this system particularly well-suited for studies of bio-interfacial phenomena include the use of surface plasmon resonance spectroscopy to measure the association of proteins with monolayers (3) and of microcontact printing to pattern the formation of monolayers (7).

This chapter reviews our work that has used monolayers presenting oligo(ethylene glycol) groups to control the interaction of proteins and cells with interfaces. The chapter begins with an introduction to SAMs, then discusses the

properties of SAMs presenting oligo(ethylene glycol) groups, the interactions of proteins with functionalized SAMs, and methodologies that use techniques from microfabrication to create substrates that control the attachment of cells.

Self-Assembled Monolayers of Alkanethiolates on Gold. Self-assembled monolayers of alkanethiolates on gold form upon the adsorption of long chain alkanethiols, RSH [R = X(CH₂)_n, n = 11-18] from solution (or vapor) to a gold surface:



Extensive experimental work has shown that the sulfur atoms coordinate to the three-fold sites of the gold(111) surface and the close-packed alkyl chains are trans-extended and tilted approximately 30° from the normal to the surface (Figure 1). The terminal functional group X is presented at the surface and determines the properties of the interface. The properties of SAMs can be controlled further by formation of "mixed" SAMs from solutions of two or more alkanethiols.

Kinetics of Formation of Monolayers. The mechanisms for the assembly of monolayers are complex and not completely understood. Several groups have studied the kinetics for assembly of alkanethiolates on gold (8-11). This work has used different methods, and although the data are not entirely consistent, most indicate that greater than 90% of the monolayer forms quickly—within minutes for mM solutions of thiol—and the remainder forms more slowly over hours. The kinetics for the initial, rapid assembly of the monolayer are probably dominated by the interaction between the thiolate and gold substrate and gives a monolayer that is locally ordered but contains defects. We presume that the second, slower phase of assembly involves the reordering of alkanethiolates on the surface and transfer of alkanethiol molecules from solution to the remaining vacant sites on the gold substrate.

For many terminal groups, the differences in properties of the monolayers formed under different conditions are minor; the contact angle of water on SAMs of octadecanethiolate, for example, is insensitive to the differences in structure of the phases formed in the terminal stages of assembly (9). For SAMs presenting other groups, however, the properties can change dramatically with increasing density of alkanethiolates. In a subsequent section we describe differences in the adsorption of protein to SAMs presenting oligo(ethylene glycol) groups that depend on the preparation of the monolayers.

Theory of the Mechanisms Underlying the Ability of PEG to Resist Adsorption of Protein. In aqueous solution, poly(ethylene glycol) chains are solvated and disordered; measurements using NMR spectroscopy (12) and differential thermal analysis (13) indicate that as many as three water molecules are associated with each repeat unit. Further evidence for the large excluded volume of PEG comes from gel chromatography experiments that show PEGs are substantially larger than other polymers of similar molecular weight (14). De Gennes and Andrade have proposed that surfaces modified with long PEG chains resist the adsorption of protein by "steric stabilization" (15,16). Adsorption of protein to the surface causes the glycol chains to compress, with concomitant desolvation. The energetic penalty of transferring water to the bulk and the entropic penalty incurred upon compression of the layer both serve to resist protein adsorption.

Analytical Methods that Measure Adsorption. Experimental studies of protein adsorption require analytical methods that can measure adsorption with high sensitivity; for example, 10% of a monolayer of a globular protein having a molecular weight of 30 kD corresponds to a density of ~0.3 ng/mm². It is also preferable that the techniques measure adsorption in real time to provide kinetic data

and are non-invasive in that they do not affect or damage the layer of adsorbed protein. Several groups have used techniques based on ellipsometry (1,2), quartz crystal microbalance (17), surface acoustic waves (17), waveguide interferometry (19), and surface plasmon resonance (SPR) (3). Ellipsometry remains a convenient technique for measuring the amount of protein on substrates that had been removed from solution and dried; it is less convenient for *in situ* measurements. SPR fulfills all of these criteria and has the additional advantage that instruments are now available from commercial vendors. Because SPR itself uses thin films of gold, it is well-suited for characterizing adsorption on monolayers of alkanethiolates.

SAMs Presenting Oligo(ethylene glycol) Groups Resist the Non-specific Adsorption of Protein. We have used monolayers that present short oligomers of the ethylene glycol group to investigate the basis for PEG to resist the adsorption of protein (1-3). Our work has used mixed SAMs prepared from solutions containing a functionalized alkanethiol ($\text{HS}(\text{CH}_2)_{11}(\text{OCH}_2\text{CH}_2)_n\text{OH}$, $n=2-6$) and a methyl-terminated alkanethiol ($\text{HS}(\text{CH}_2)_{10}\text{CH}_3$). This system permits control over both the length and density of glycol chains at the surface (Figure 2). Because adsorption can depend dramatically on the structure of a protein (19), we have used a panel of representative proteins to characterize these monolayers. Both *ex situ* ellipsometry and *in situ* SPR show that SAMs presenting only oligo(ethylene glycol) groups resist almost entirely the adsorption of protein. Extensive work in our laboratory shows that these surfaces are very effective in resisting adsorption of proteins, and even resist adsorption from concentrated (1-10 mg/mL) solutions of mixtures of protein. Mixed SAMs presenting this group together with as much as 50% hydrophobic, methyl groups also resist the adsorption of protein; SAMs presenting methyl groups alone adsorb most proteins rapidly and irreversibly. The ability of the surface to resist adsorption increases with both the density and length of the oligomer.

Mechanisms of Inert SAMs. Our work shows that surfaces presenting densely packed short oligomers of the ethylene glycol group are highly effective at resisting the adsorption of protein. It is not clear that the mechanisms for this resistance of SAMs presenting short, oligo(ethylene glycol) chains are similar to those for high molecular weight PEG. The extensive solvation of PEG by water molecules is almost certainly critical to the properties of the bulk polymer, but SAMs presenting dense packed oligo(ethylene glycol) groups probably do not have sufficient volume to accommodate extensive solvation. Molecular modeling studies even suggest that the perfectly ordered SAMs cannot include any solvent (20). Because the glycol chains in the monolayers are each covalently tethered to the surface, these thin films should have conformational properties very different from those of the unconstrained bulk polymer.

Recent work from our laboratory, and that of Professor Michael Grunze at Heidelberg, have shown that the conditions used to prepare the SAMs are critical to the interfacial properties. SAMs prepared from solutions of a hexa(ethylene glycol)-terminated alkanethiol for periods of less than 12 hr—the usual conditions—resist the adsorption of protein. SAMs prepared from these same solutions, but allowing the equilibration of the structure of the SAMs to proceed for periods of 1-7 days in contact with the solution of thiol, are less effective in resisting the adsorption of protein; the amount of protein that adsorbs irreversibly to these surfaces increases with the time over which formation of the SAM is allowed to occur.

One explanation for these data is that the final stage of the assembly of alkanethiols substituted with oligo(ethylene glycol) groups onto a gold surface is slow, and that the interfacial properties depend strongly on the density of alkanethiolates in the monolayer. Immersion times of 12 hr may give SAMs that still have a substantial number of vacant coordination sites on the gold surface—we have no experimental data to suggest what this critical density of holes may be—and a surface that resists adsorption of protein. Longer immersion times may give SAMs having fewer vacant coordination sites and defects, and that adsorb protein. Other

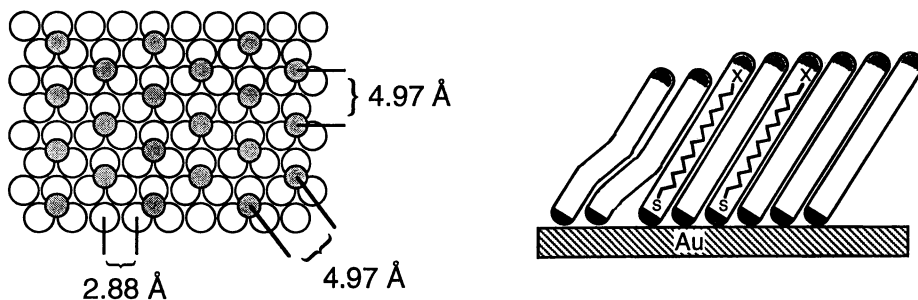


Figure 1. Representation of a self-assembled monolayer (SAM) of alkanethiolates on the surface of gold. (Left) Hexagonal coverage scheme of thiols coordinated to the gold (111) surface; the sulfur atoms (shaded circles) fill the hollow three-fold sites on the gold surface (open circles). (Right) The alkyl chains are close-packed and tilted approximately 30° from the normal to the surface. The properties of the SAM are controlled by changing the length of the alkyl chain and the terminal functional group X of the precursor alkanethiol. The missing row represents a common defect present in SAMs. The detailed structures of point and line defects have not been established.

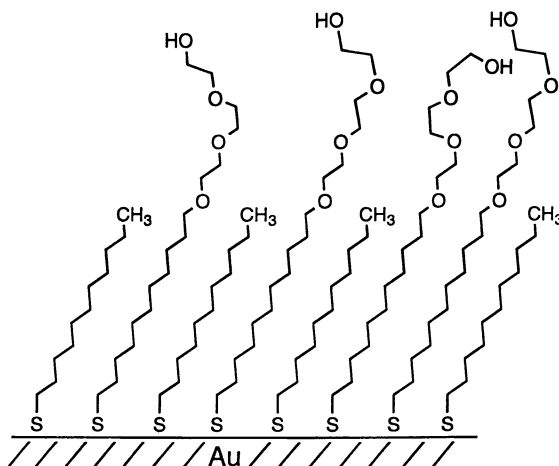


Figure 2. Representation of a mixed SAM terminated in methyl groups and tri(ethylene glycol) groups. We presume that the polymethylene chains are more ordered than the glycol groups. The density of the tri(ethylene glycol) groups at the surface is determined by the ratio of the two alkanethiols in the solution from which the SAM is formed.

factors that may be important in understanding the mechanisms for adsorption are the conformation of the oligo(ethylene glycol) groups in the monolayers and the role of other defects (for example, those produced by corrosion of the gold substrate). We are currently investigating this system in greater detail.

Design of Surfaces that Resist Adsorption. We sought to design a new material that shared the properties of PEG to resist the adsorption of protein. We chose the propylene sulfoxide group because oligomers of this group, like those of ethylene glycol, are hydrophilic, well-solvated by water and conformationally flexible. We prepared monolayers presenting tri(propylene sulfoxide) groups ($\text{HS}(\text{CH}_2)_{11}(\text{SOCH}_2\text{CH}_2\text{CH}_2)_3\text{SOCH}_3$) (Figure 3). SPR showed that these SAMs resisted the non-specific adsorption of fibrinogen, RNase A, and other proteins (21). These SAMs remained inert to adsorption even when mixed with as much as 50% methyl-terminated alkanethiolate. Although the different lengths of these two groups prevents a direct comparison, the data suggest that when presented at an interface they are similarly effective at resisting adsorption. The most important result from this work is the demonstration of a successful process—that is centered on the combination of SAMs and SPR—for the *de novo* design and testing of a new inert material. This study also suggests that PEG is not unique in its ability to serve as an inert surface, but that there will probably be many such polymers.

The inert surfaces provided by these monolayers serve as the basis for the design of biointerfaces having other properties. For example, ligands can be immobilized to these SAMs to create substrates that bind a specific receptor yet still resist the non-specific adsorption of other proteins. These inert monolayers also make possible a convenient and flexible methodology for creating patterned substrates that control the attachment of mammalian cells. The remainder of this chapter describes our work in these areas.

Bio-Specific Adsorption. The bio-specific adsorption of proteins to surfaces presenting appropriate ligands is important in drug screening, cell culture, biosensing, and other areas. These applications have also used empirical approaches and few studies have investigated fundamental aspects of biomolecular recognition at surfaces. The most serious problem encountered with surfaces designed for bio-specific adsorption is the non-specific adsorption of other proteins to the surface. The common strategy of coating a material with serum albumin, for example, suffers from poor reproducibility in the adsorption and from limited stability of the protein layer (22).

We have used SAMs presenting tri(ethylene glycol) groups and benzensulfonamide groups as model substrates with which to study the bio-specific adsorption of carbonic anhydrase (23). SPR showed that the protein bound reversibly to these SAMs and provided kinetic rate constants for association and dissociation (Figure 4). The binding of protein was bio-specific; addition of a soluble ligand of the CA to the protein-containing solution prior to the binding experiment inhibited adsorption of the protein to the surface. The amount of protein that bound at saturation increased with the density of the ligand on the surface; this density could be controlled by adjusting the ratio of the two alkanethiols in the solution from which the monolayer assembles. When a complex mixture containing nine proteins (2 mg/mL total concentration) was introduced into the flow cell, SPR recorded essentially no protein adsorption; however, when CA was present in this complex sample, SPR measured binding of the protein with no complications due to the other proteins (Figure 4). This system provides a convenient model for biophysical studies of biointerfacial recognition.

Chemo-Specific Adsorption. We have demonstrated a related immobilization strategy based on the well-known coordination of oligo(histidine) peptides by complexes of nickel (II) (24). Mixed SAMs presenting nitrilotriacetic acid (NTA)

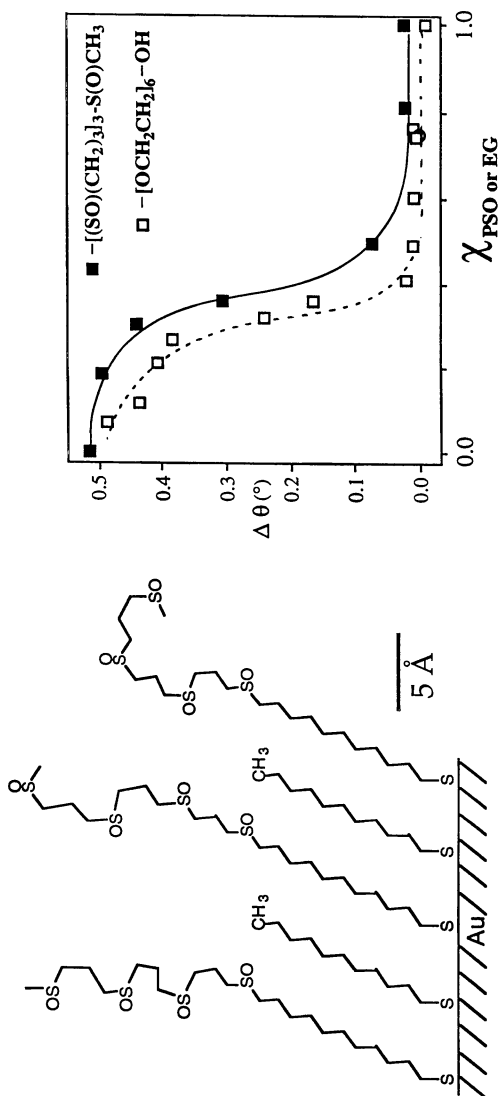


Figure 3. (Left) Representation of a mixed SAM terminated in methyl groups and tri(propylene sulfide) groups; the proportion of functionalized alkanethiolate is given by χ . The plot on the right compares the adsorption of fibrinogen on these SAMs with SAMs terminated in hexa(ethylene glycol) groups. The amount of protein that adsorbed to the monolayers was measured using surface plasmon resonance spectroscopy and is reported as a change in resonance angle ($\Delta\theta$) upon binding (3, 21).

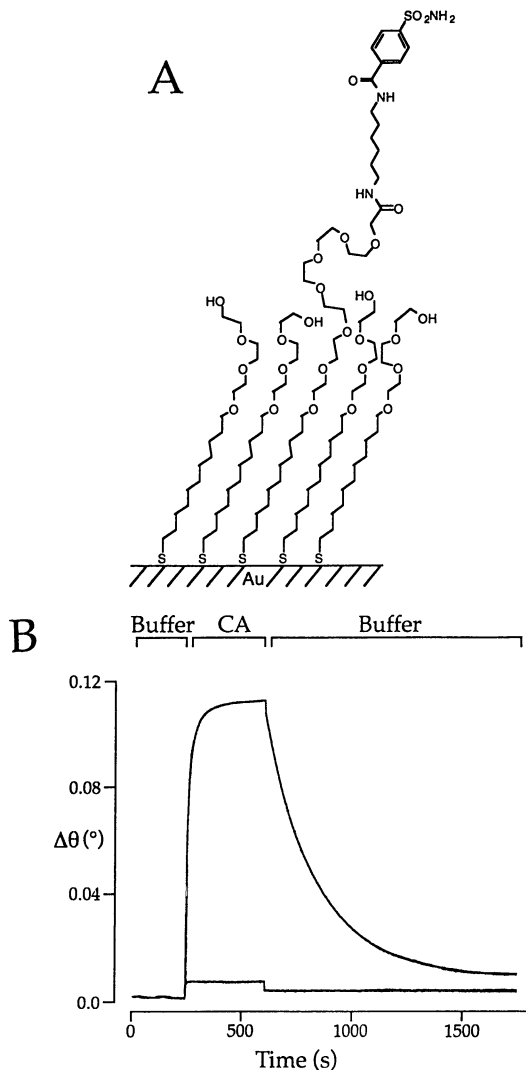


Figure 4. SPR was used to measure the rate and quantity of binding of carbonic anhydrase (CA) to a SAM terminated in EG₃ groups and benzenesulfonamide groups (A). The change in resonance angle ($\Delta\theta$) of light reflected from the SAM/gold is plotted against time; the time over which the solution of CA (5 μM) was allowed to flow through the cell is indicated at the top of the plot (B). The upper curve shows binding (and dissociation) of CA to a SAM containing ~5% of the ligand-terminated alkanethiolate. CA did not adsorb to a SAM presenting only ethylene glycol groups (lower curve). A response due to the change in index of refraction of the CA-containing solution was observed upon introduction of protein into the flow cell (evident in the lower curve). The difference between the measured response and this background signal represents binding of the CA to the SAM.

chelates of Ni(II) and tri(ethylene glycol) groups were used to capture histidine-tagged recombinant proteins from cell extracts (Figure 5). Only the his-tagged proteins adsorbed to the SAM; the other proteins in the sample did not interfere with the coordination nor did they adsorb to the SAM. This immobilized, his-tagged protein was stable but could be removed rapidly by adding imidazole as a competing ligand for the NTA group. This system has the additional advantages that the immobilized protein is presented in a single orientation and the density of protein can be controlled.

Using Microcontact Printing to Pattern Monolayers. Several groups have used photolithographic methods to pattern the formation of monolayers; these methods work well but the requirement for a lithography facility makes them inconvenient and inaccessible to many biological researchers. We have developed a new and convenient method for patterning SAMs of alkanethiols on gold with features of sizes ranging down to 1 μm (7,25,26). Microcontact printing (μCP) uses an elastomeric stamp having on its surface a pattern of relief at the micron scale (Figure 6). This stamp can be coated with a solution of alkanethiol, dried, and brought into contact with a surface of gold to transfer the alkanethiol to discreet regions of the substrate. This process produces a pattern of SAM on the gold that is identical to the pattern of relief in the stamp. A different SAM can then be formed in the remaining regions of gold by immersing the substrate in a solution of the other alkanethiol. Conformal contact between the elastomeric stamp and surface allow surfaces that are rough (at the scale of 100 nm) to be patterned over areas several cm^2 in size with edge resolution of the features better than 50 nm. Multiple stamps can be cast from a single master and each stamp can be used hundreds of times. Microcontact printing has been used to pattern SAMs of alkylsiloxanes on oxide substrates (27) and can even form patterns on curved substrates (28).

Patterning the Adsorption of Protein. Microcontact printing can prepare substrates that adsorb protein in patterns. The method begins by contact printing a SAM of hexadecanethiolate on a gold substrate to give a pattern of hydrophobic, methyl-terminated SAM. Rinsing this substrate in a solution of oligo(ethylene glycol)-terminated alkanethiol renders the remaining regions of gold inert to protein adsorption. Immersion of the patterned substrate in a solution of protein results in the rapid and irreversible adsorption of protein to the hydrophobic, methyl-terminated regions of the monolayer (Figure 7). Scanning electron microscopy provides a convenient method for imaging the patterned protein (29). This method is experimentally simple and can pattern proteins at the micron scale. It has the limitation that it cannot pattern the adsorption of multiple proteins to a single substrate. Photolithographic methods that combine immobilization chemistries have been used to pattern the formation of multiple proteins on a single substrate (30).

Patterning the Attachment of Cells on Planar Substrates. This same methodology for patterning the adsorption of protein can be used to prepare substrates for patterning the attachment of mammalian cells (31,32). For attachment to surfaces, cells use membrane receptors to recognize immobilized ligands normally found in the extracellular matrix proteins. Consequently, surfaces presenting a pattern of a matrix protein will direct the attachment and spreading of cells, provided that the intervening regions of surface are inert to attachment. We have prepared substrates containing a pattern of adsorbed fibronectin (the most common matrix protein) and oligo(ethylene glycol) groups. Addition of a suspension of capillary endothelial cells to the substrate resulted in the attachment of cells only to the protein-coated regions (Figure 8). The spreading of the attached cells was also confined to the underlying pattern of protein (and SAM). This methodology was also used to pattern the attachment of *individual* hepatocytes (31).

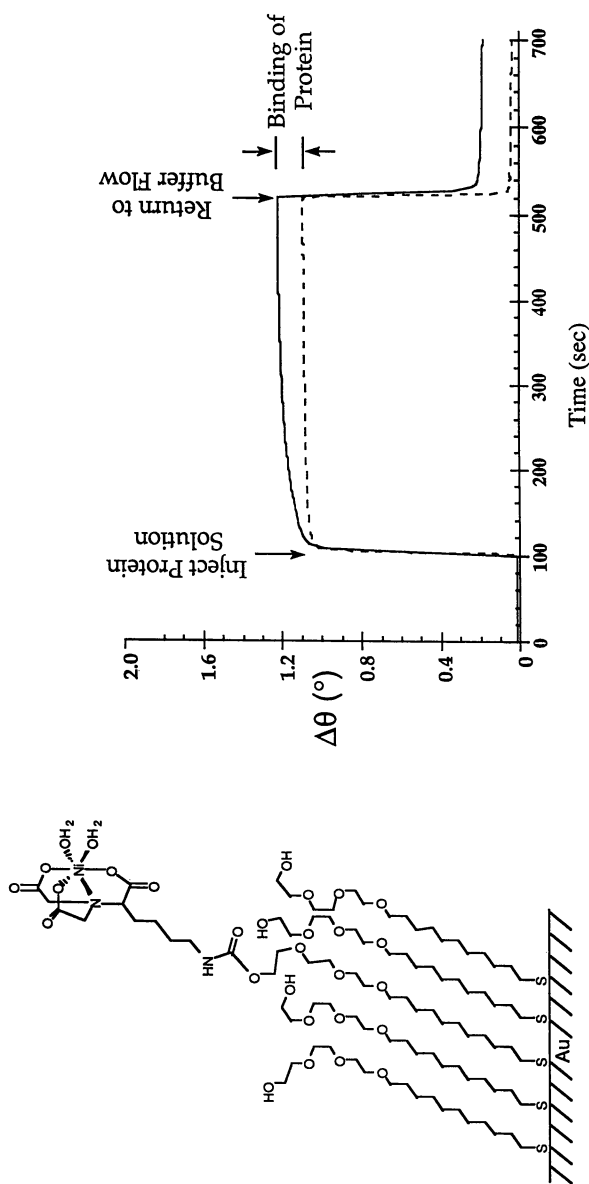


Figure 5. SPR was used to measure the rate and quantity of binding of a His-tagged T-cell receptor construct to a SAM terminated in EG₃ groups and Ni(II) complexes (24). (Left) The mixed SAM contains ~ 5% of the Ni(II)-functionalized alkanethiol. Imidazole rings of the His-tagged protein replace the water ligands of the Ni(II) complex. (Right) The change in resonance angle ($\Delta\theta$) of light reflected from the SAM/gold is plotted against time. A large response due to the change in index of refraction of the solution was observed upon introduction of protein into the flow cell (denoted by the solid curve). The difference between the measured response and this background signal represents binding of the His-tagged protein to the SAM.

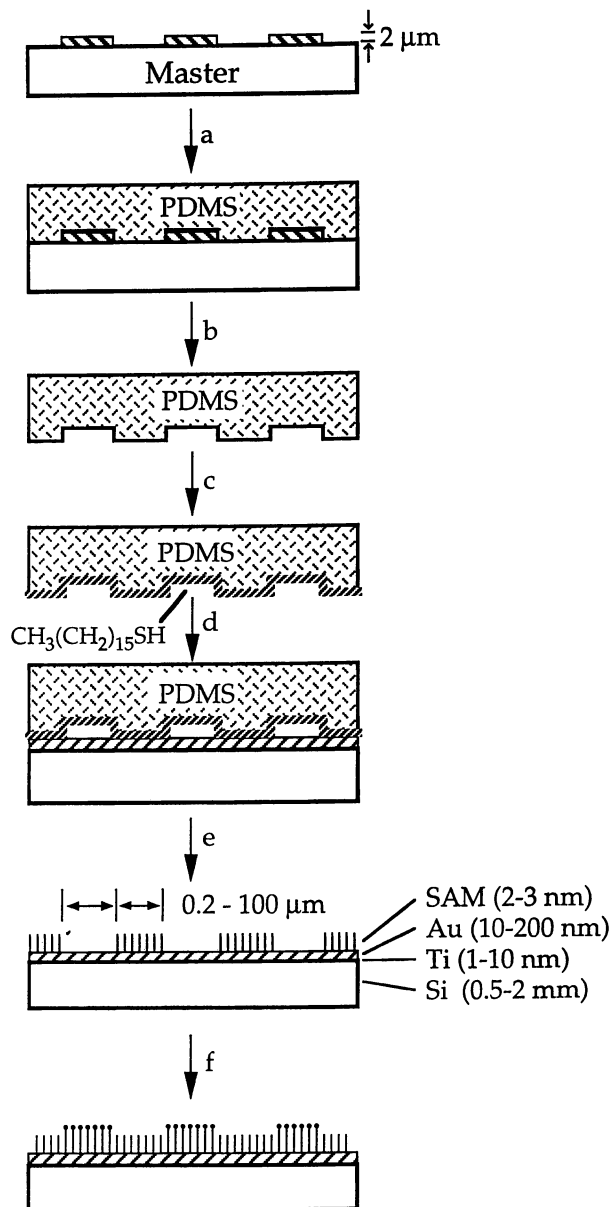


Figure 6. Microcontact printing starts with a master template containing a pattern of relief (a); this master can be fabricated by photolithography, or other methods. A polydimethylsiloxane (PDMS) stamp cast from this master (b) is "inked" with a solution of alkanethiol in ethanol (c) and used to transfer the alkanethiol to surface of gold (d); a SAM is formed only at those regions where the stamp contacts the surface (e). The bare regions of gold can then be derivatized with a different SAM by rinsing with a solution of a second alkanethiol (f).

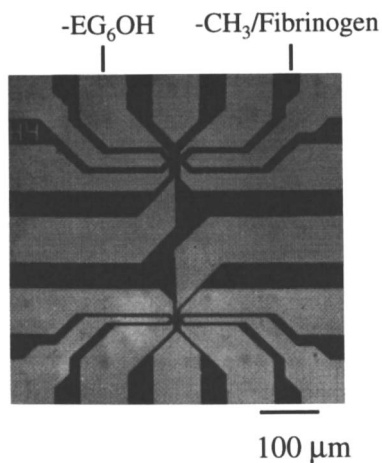


Figure 7. Scanning electron micrographs of fibrinogen adsorbed on a patterned SAM. A patterned hexadecanethiolate SAM on gold was formed by microcontact printing and the remainder of the surface was derivatized by exposure to a hexa(ethylene glycol)-terminated alkanethiol ($\text{HS}(\text{CH}_2)_{11}(\text{OCH}_2\text{CH}_2)_6\text{OH}$). The patterned substrate was immersed in an aqueous solution of fibrinogen (1 mg/mL) for 2 hr, removed from solution, rinsed with water, and dried. Fibrinogen adsorbed only to the methyl-terminated regions of the SAM, as illustrated by the dark regions in the SEM micrograph: secondary electron emission from the underlying gold is attenuated by the protein adlayer.

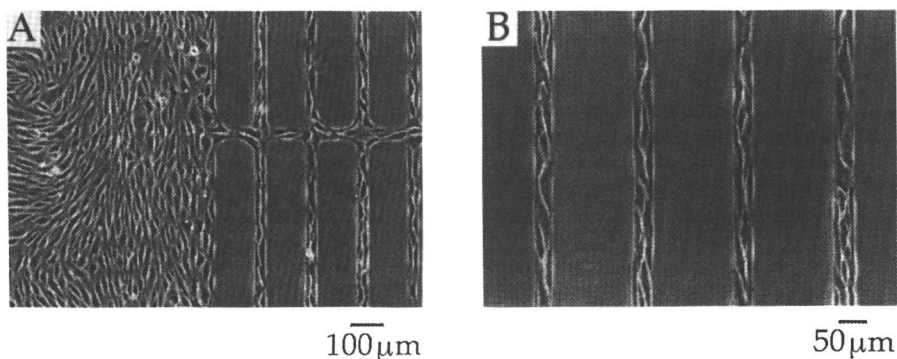


Figure 8. Control over the attachment of bovine capillary endothelial cells to planar substrates that were patterned into regions terminated in methyl groups and tri(ethylene glycol) groups using μCP . The substrates were coated with fibronectin prior to cell attachment; fibronectin adsorbed only to the regions of methyl-terminated SAM. (A) An optical micrograph showing attachment of endothelial cells to a non-patterned region (left) and to lines 30 μm in width. (B) A view at higher magnification of cells attached to the lines.

Controlling the Attachment of Cells on Contoured Substrates. We have combined this methodology with techniques for microfabrication to prepare contoured substrates that direct the attachment of cells (33). An elastomeric stamp was used to mold a film of polyurethane into alternating grooves and plateaus 50 μm in width. A thin, optically-transparent film of gold was evaporated onto this substrate on which monolayers could be formed. A flat PDMS stamp was used to form a SAM of hexadecanethiolate on the raised plateaus of the contoured surface by contact printing hexadecanethiol and a SAM terminated in tri(ethylene glycol) groups was subsequently formed on the bare gold remaining in the grooves by immersing the substrate in a solution of a second alkanethiol. Figure 9 shows that endothelial cells attached and spread only on the hydrophobic regions of the substrate that presented fibronectin.

Conclusions

The work described in this chapter presents a comprehensive methodology suitable for the study of biointerfacial phenomena. The flexibility offered by self-assembled monolayers to tailor the properties of an interface and present biologically relevant groups—including molecules, peptides and proteins—provides an opportunity to understand, in detail, the relationship between interfacial structure and properties. A range of analytical techniques, and surface plasmon resonance in particular, provide a methodology to understand the interactions and dynamics of surfaces with proteins and cells. Microcontact printing and related techniques for microfabrication make possible the design of a range of substrates with which to control and understand the biological responses to materials. This combination of techniques has already made possible new types of experiments relevant to biosurfaces and will certainly be important in work that follows.

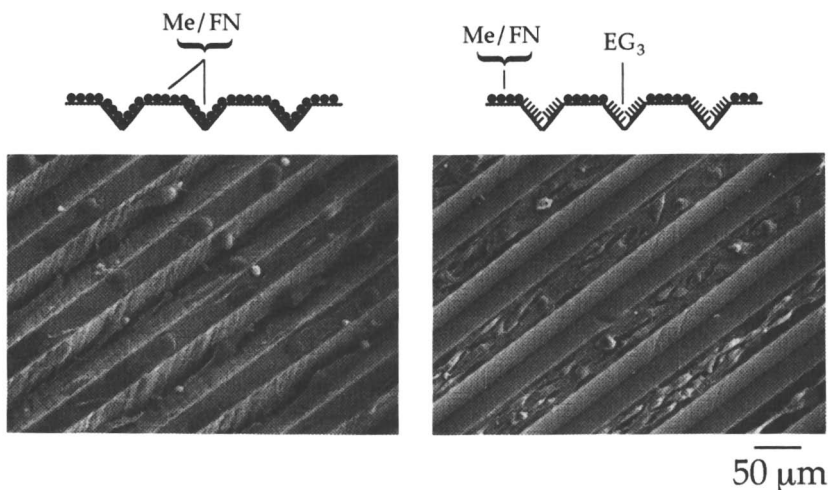


Figure 9. Control over the attachment of endothelial cells to contoured surfaces using self-assembled monolayers. The substrates are films of polyurethane (supported on glass slides) that were coated with gold and modified with SAMs of alkanethiolates terminated in methyl groups and tri(ethylene glycol) groups; the substrates were coated with fibronectin prior to cell attachment. (Left) Cells attached to both the ridges and grooves of substrates presenting fibronectin at all regions. (Right) Cells attached only to the ridges when the grooves were modified with a SAM presenting tri(ethylene glycol) groups.

Acknowledgments

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Harris and Zalipsky; Poly(ethylene glycol)