Using Self-Assembled Monolayers to Study the Interactions of Man-Made Materials with Proteins

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Overview

The first event that occurs on contact of a synthetic material with a medium containing dissolved protein--such as blood or plasma--is the adsorption of protein to the surface. Other responses, including the attachment of cells and organization of tissue, are secondary and depend on the nature of the adsorbed layer of protein. Despite the identification of many surfaces suitable for biomedical applications, the molecular details of the processes by which proteins adsorb to solid surfaces are not completely understood. This understanding has been limited in large part by a lack of well-defined surfaces whose properties could be tailored at the molecular scale. Self-assembled monolayers (SAMs) constitute a class of model surfaces particularly well suited for studying interactions of proteins and cells with surfaces. The ability to control the composition and properties of SAMs--particularly SAMs formed upon the absorption of long-chain alkanethiols on gold--through synthesis combined with simple methods for patterning their functional groups in the plane of the monolayer, make this class of surfaces the best currently available for fundamental molecular mechanistic studies of protein adsorption and cell adhesion. This chapter reviews the use of two classes of SAMs--alkanethiolates on gold and alkylsiloxanes on surfaces presenting hydroxyl groups--for studies of protein adsorption and cell attachment.

Background

Protein Adsorption. The interactions of proteins with man-made surfaces have been studied extensively;[1-6] early studies were motivated by the development of blood compatible materials and devices.[7] The process of adsorption is complicated and depends on the characteristics of the protein, the interface and the solution. Even in the case of a single protein adsorbing to a structurally-defined and homogeneous surface, the protein may adsorb in multiple orientations and conformations and in differing microenvironments created by neighboring proteins (Fig. 1). A recent report describing

Figure 1. Scheme illustrating the complexities associated with studies of protein adsorption. A protein that absorbs to the surface may also desorb with no change in its structure and function (a). The adsorbed protein may diffuse on the surface (b) and then desorb (c), or undergo a conformational change (d) and desorb (e) or remain irreversibly adsorbed (f). In some cases, the adsorbed protein may be displaced from the surface by another protein in solution (g; the "Vroman effect," [7, 20]). This scheme is not complete, but is further complicated by the many different orientations, conformations, and environments available to an adsorbed protein.

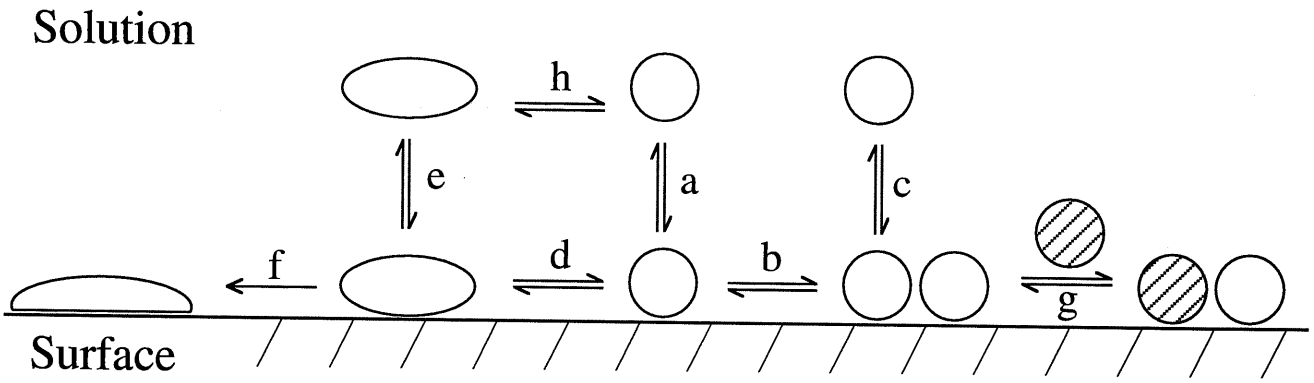


Fig. 1

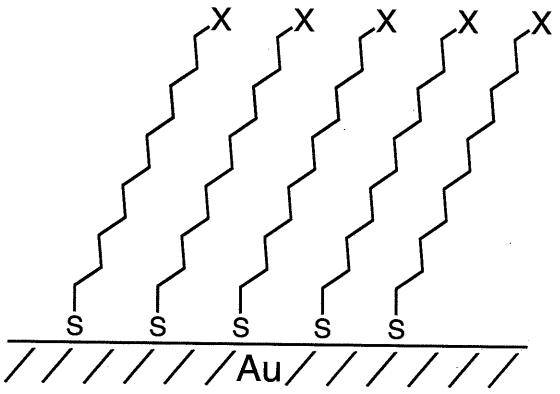
the changes in adsorption kinetics of the soluble core tryptic fragment of cytochrome *b5* due to a single amino acid substitution emphasizes the need to understand protein adsorption at the molecular level.[8]

Methods to Characterize Protein Adsorption. The amounts and rates of protein adsorption have beeen determined by techniques that measure the dielectric properties at an interface [surface plasmon resonance (SPR) spectroscopy,[9,10] waveguide interferometry,[11] and ellipsometry[12]] or changes in the resonance frequency of piezoelectric materials.[13] Although these techniques give no direct information regarding the orientation and structure of adsorbed protein, they provide information that is qualitatively related to conformational changes of adsorbed proteins. Lee and Belfort interpreted the increase in the enzymatic activity of RNase A adsorbed on mica with increasing residence times in terms of a reorientation of the adsorbed protein that made the active site more accessible to the solution.[14] Conformational changes in BSA adsorbed to fluoropolymer surfaces were correlated to cell adhesion using fluorescent tagging methods.[15] Spectral techniques that are common in characterizing soluble proteins--such as circular dichroism[16,17] and infrared spectroscopies[18]--are limited by their sensitivity for studying adsorbed proteins.

Molecular-Scale Characterization. Robertson and coworkers used a panel of monoclonal antibodies having defined epitopes against myoglobin to probe the orientation of the protein adsorbed to mica surfaces.[19] Vroman and colleagues used similar antisera methods in pioneering studies of adsorption of serum proteins to biomaterials.[7, 20] This strategy, and related "footprinting" strategies[21]--using either chemical reagents or proteases--provide direct, but low resolution, information about protein topology at surfaces. For special cases where the protein layer is ordered, other techniques can provide direct structural information: Caffrey and coworkers, for example, used X-ray

Figure 2. Representations of self-assembled monolayers (SAMs) of alkanethiolates on gold (A) and alkylsiloxanes on hydroxylated surfaces (B). The alkyl chains of the alkanethiolates pack in a quasi-crystalline array; the alkyl chains of the alkylsiloxanes are less ordered. The properties of both classes of SAMs are determined primarily by the terminal functional group X.

A



B

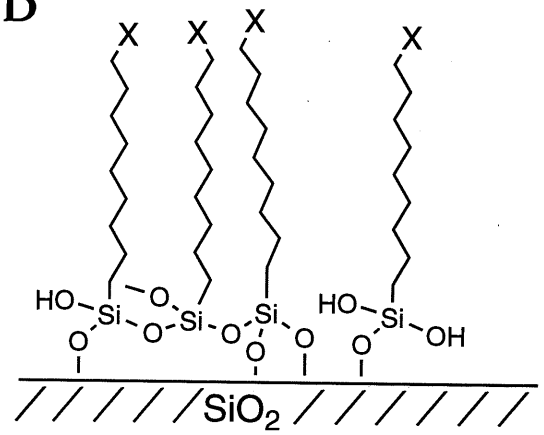


Fig 2

standing wave methods to characterize the orientation of cytochrome c adsorbed to a silver surface;[22] Kornberg and coworkers used electron diffraction to determine the structure of a two-dimensional crystal of streptavidin adsorbed to a biotinylated lipid layer.[23]

Self-Assembled Monolayers

Alkanethiolates on Gold. Self-assembled monolayers of alkanethiolates on gold form upon the adsorption of long chain alkanethiols, RSH [R = $X(CH_2)_n$, n = 11-18] from solution (or vapor) to a gold surface:

$$RSH + Au(0)_n \text{----->} RS^-Au(I)\bullet Au(0)_n + 1/2\ H_2(?) \qquad \text{(eq 1)}$$

The structure of these SAMs is well established (Fig. 2).[24,25] The sulfur atoms coordinate to the three-fold sites of the gold(111) surface. The close-packed alkyl chains are trans-extended and tilted approximately 30° from the normal to the surface. The terminal functional group X is presented at the surface and determines the properties of the interface. Even complex groups can be incorporated through modest synthetic effort; alternatively, groups can be introduced onto the surface after the SAM is formed. The properties of SAMS can be controlled further by formation of "mixed" SAMs from solutions of two or more alkanethiols. SAMs on gold are stable in air or in contact with water or ethanol for periods of several months; the thiolates desorb at temperatures greater than 70°C and are photo-oxidized when irradiated with UV light in the presence of oxygen. SAMs have sufficient stability in aqueous media for use in cell culture for periods of days. The optical transparency of the system of SAMs on gold depends on the thickness of the underlying gold; gold films 100 Å in thickness are optically transparent; gold films 2000 Å in thickness are opaque and reflective. Even the thinner films of gold are electrically conductive.

Alkylsiloxanes. Alkylsiloxane monolayers are formed by reaction of a hydroxylated surface (usually glass or silicon oxide) with a solution of alkyltrichlorosilane (or alkyltriethoxysilane).[26-27] The structure of these monolayers is not as well established as those of alkanethiolates on gold, and depends on the conditions used to prepare the SAMs.[26, 28] The siloxane groups cross-link with each other and with hydroxyl groups of the surface, though the relative importance of the two interactions is still not completely understood.[29] Alkyl siloxane SAMs are significantly more stable thermally than alkanethiolates on gold and do not require the deposition of a layer of metal for the preparation of substrates. They have the disadvantages that they are structurally less ordered than alkanethiolates on gold and are limited in the range of functional groups that can be incorporated directly--without carrying out reactions at the surface--by the reactivity of the alkyltrichlorosilane precursors.

Interactions of Proteins with SAMs

Non-specific adsorption. Hydrophobic forces appear to dominate the interactions of proteins with surfaces in many systems. Many studies have found that the adsorption often increases with the hydrophobicity of both the surface and the protein.[30,31] The amount of several proteins adsorbing to SAMs on gold presenting a wide range of functional groups correlated with the hydrophobicity of the SAM.[32] The protein layer is usually irreversibly bound to hydrophobic surfaces, but can be removed with detergents, or by exchange with other proteins in solution--the "Vroman effect."[20] The role of electrostatic interactions in adsorption has been less well studied. In a recent study using SAMs with charged functional groups, Tengvall and coworkers showed that the adsorption of each of five serum proteins showed a different dependence on type of surface charge.[33]

Protein-Resistant Materials. An important goal in biomaterials is the development of "inert" materials that resist protein adsorption. A common method to passivate surfaces towards protein adsorption is to modify the surface with poly(ethylene glycol) (PEG); several strategies have been devised to immobilize this polymer at surfaces.[34-36] Another common strategy is to coat the surface with a protein, usually bovine serum albumin, that resists the adsorption of other proteins. This latter method has the advantages that it is simple and inexpensive, but suffers from limited stability of the protein layer owing to exchange with other proteins in solution, and presentation of biologically active peptide sequences.

SAMs Presenting Ethylene Glycol Groups. We found that SAMs on gold prepared from alkanethiols terminated in short oligomers of the ethylene glycol group [$HS(CH_2)_{11}(OCH_2CH_2)_n$, n = 2-7] resisted entirely the adsorption of several proteins.[37,38] Even SAMs containing as much as 50% hydrophobic methyl-terminated alkanethiolates, if mixed with oligo(ethylene glycol)-terminated alkanethiolates, resisted the *in situ* adsorption of protein. The basis for protein resistance in this system is not well understood. DeGennes and Andrade have proposed that surfaces modified with *long* PEG chains resist the adsorption of protein by "steric stabilization;" where adsorption of protein to the surface causes the solvated and disordered glycol chains to compress; the energetic penalty of desolvating the glycol chains and restricting the conformational freedom of the chains both serve to resist adsorption. It is unclear whether this mechanism applies to *short*, densely-packed oligo(ethylene glycol) chains.[39]

Covalent Immobilization of Proteins to SAMs. Methods that use covalent immobilization to confine proteins at interfaces have many advantages over those that rely on physical adsorption of protein layers, especially when well-defined surfaces are employed. Covalently attached layers of protein cannot dissociate from the surface, or exchange

with other proteins in solution. A variety of selective chemistries can be employed to provide high levels of control over the adsorption process. For example, Bohn, Sligar, and coworkers used genetic engineering to construct a mutant of cytochrome c that had only a single cysteine group; immobilization of this protein to a SAM terminated in thiol groups gave a uniformly oriented layer of protein.[40]

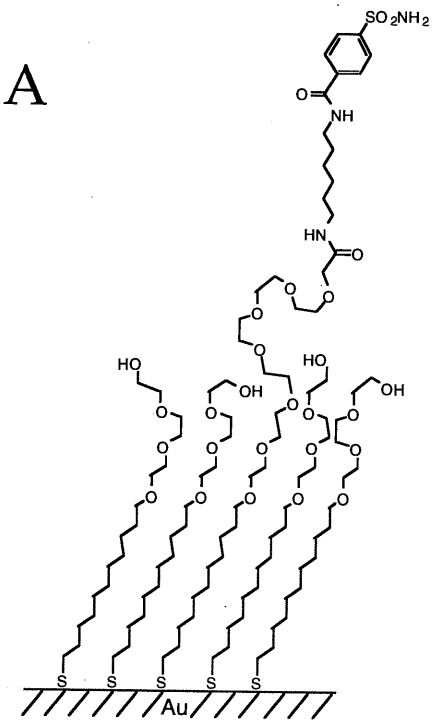
Bio-Specific Adsorption of Protein. The synthetic flexibility offered by SAMs of alkanethiolates make these surfaces useful for studies of bio-specific adsorption. Several groups have characterized the recognition of antigens immobilized on SAMs by antibodies.[41] Spinke et al used SPR to study the specific binding of streptavidin to SAMs terminated in biotin groups.[42] We have used the combination of SPR and SAMs presenting oligo(ethylene glycol) groups as supports to which ligands were attached for studies of reversible bio-specific recognition by proteins. SAMs presenting a Ni(II) complex selectively bound proteins whose primary sequence terminated with a histidine tag, a modification often incorporated into recombinant proteins to facilitate purification.[43] In a similar system, SPR was used to characterize the binding of carbonic anhydrase to SAMs presenting benzenesulfonamide groups (Fig. 3).[44] In both cases, the amount of adsorbed protein increased with density of the ligand on the surface and the surfaces resisted non-specific adsorption of other proteins. These SAM systems are especially well-suited for fundamental studies of bio-molecular recognition at surfaces because both the density and environment of immobilized ligands can be controlled.

Attachment of Cells to SAMs

The attachment and spreading of anchorage-dependent cells is mediated by proteins of the extracellular matrix (ECM); e.g., fibronectin, laminin, collagen and others. Bio-specific recognition of peptide sequences of the ECM by membrane proteins of the cells is important in these processes; the best understood of these interactions is the binding of

Figure 3. Surface plasma resonance spectroscopy measures the rate and quantity of binding of carbonic anhydrase (CA) to a mixed SAM presenting oligo(ethylene glycol) and benzenesulfonamide groups (A). The change in resonance angle ($\Delta\theta$) of reflected light from gold interface is shown as a function of time (B). The mixed SAM resists the non-specific adsorption of protein from a solution containing β-casein, myoglobin, alcohol dehydrogenase, trypsin inhibitor, acylase I, α-lactalbumin, cytochrome c, fibrinogen, and RNase A (0.2 mg/ml each; these proteins were chosen arbitrarily from those readily available, and were intended only to test specificity) (bottom curve). When CA (5 mM) was present in this sample, however, SPR measured adsorption of the protein (upper curve).

A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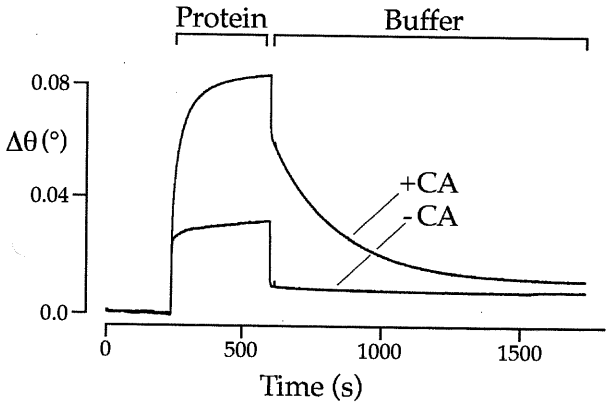
Protein
Buffer
0.08
Δθ (°)
0.04
0.0
+CA
-CA
0
500
1000
1500
Time (s)

Fig. 3

the peptide sequences RGD and YIGSR by cellular integrins. Consequently, the identity of the protein layer, and the molecular conformations of the proteins within the layer are important for cell attachment. Both hydrophobic[45] and ionic[46] SAMs have been used as substrates for cell culture. A significant problem with these preparations is the lack of control over the adsorption process. Studies of the differentiation response of fibroblasts and neuoroblastoma cells on siloxane SAMs terminated in different groups coated with fibronectin showed that cell behavior depends upon the conformation of fibronection and not the density of protein on the surface.[18, 47]

Patterning the Formation of SAMs.

Microcontact Printing. Microcontact printing (μCP)[48-50] is a convenient method that can pattern SAMs of alkanethiolates on gold in the plane of the monolayer, with features down to 1 μm conveniently, and to 200 nm in special cases (Figure 4).[51] Microcontanct printing uses an elastomeric stamp that is formed by casting polydimethylsiloxane (PDMS) against an appropriate relief structure, usually a photolithographically produced master. The PDMS stamp is inked with a solution of the alkanethiol in ethanol, dried, and manually brought into contact with a gold surface: the alkanethiol is transferred only at those regions where the stamp contacts the surface. Conformal contact between the elastomeric stamp and surface and the rapid reaction of alkanethiols with gold permit the surface 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 also been used to pattern siloxanes on the surfaces of SiO_2 and glass[51] and to pattern SAMs on non-planar and contoured surfaces.[52] Because μCP is a technique that relies on molecular self-assembly and does not require stringent control over the laboratory environment, it can produce μm-scale patterns conveniently and at low cost relative to methods that use photolithography.

Figure 4. Procedure for patterning SAMs using microcontact printing. Photolithography or other methods generate a mask containing features of the pattern to be reproduced. A PDMS prepolymer is poured onto the master pattern, allowed to cure (a), and peeled away from the master (b). The stamp is inked with a solution of alkanethiol (c) and used to transfer the alkanethiol to the surface (d); this transfer forms a patterned SAM (the representation of the SAM implies no structure) (e). Exposing the gold substrate to a solution of a different alkanethiol derivatizes the bare regions (f).

Photolithography. Photolithographic methods illuminate a substrate with UV light through a mask containing the pattern to be reproduced.[53] In the common "liftoff" method, a silicon oxide substrate is coated with a thin layer of photoresist and irradiated with UV light through a mask. The exposed regions are removed in a developing bath to reveal complementary patterns of silicon dioxide and photoresist. Subsequent immersion of the substrate in a solution of alkyltrichlorosilane forms siloxane SAMs. The remaining photoresist is then removed, and a different siloxane can be formed in the complementary regions. Other variants of photolithography use the UV irradiation to pattern photoreactive SAMs of siloxanes.[54, 55] The required photolithographic equipment and a controlled environment facility make this technique expensive, and substantially less convenient than μCP. The ink-jet printing of glucose oxidase on carbon electrodes to fabricate amperometric glucose biosensors[56] suggests another new and inexpensive method for patterning arrays of biomolecules.

Fabrication of Contoured Surfaces. Both μCP[57] and photolithography[58] have been used to form patterned layers (resists) that protect the substrates from dissolution in chemical etchants. Chemical etching of the patterned SAMs produces contoured features whose shapes depend on the orientation of the silicon and the duration of the etching process; etching lines in a silicon <100> surface produces V-shaped grooves. The properties of the etched substrates can then be tailored by subsequent formation of an alkylsiloxane SAM or by evaporation of a film of gold followed by formation of an alkanethiolate SAM.

Patterning the Adsorption of Protein. Patterned SAMs on gold have been used extensively to adsorb protein in patterns on surfaces (Fig. 5) This method relies on the property of SAMs terminated in oligo(ethylene glycol) groups to resist adsorption of protein. For example, μCP was used to generate regions of SAMs terminated in either

Figure 5. Scanning electron microscope (SEM) micrographs of fibrinogen adsorbed on a patterned SAM. A patterned hexadecanethiolate self-assembled monolayer (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. The patterned substrate was then immersed in a solution of fibrinogen (1 mg/ml in PBS buffer) for two hours, removed from solution, rinsed with water, and dried. Fibrinogen adsorbed only to the methyl-terminated regions of the SAM, and appear as the dark regions in the micrograph. The top image shows a pattern of the type used in the microelectronics industry. The bottom image illustrates the utility of microcontact printing for patterning the adsorption of proteins on the micron scale. Reprinted with permission from: Mrksich M, Whitesides GM. Patterning self-assembled monolayers using microcontact printing: a new technology for biosensors? Trends in Biotechnology 1995; 13:228-235. Copyright 1995 Elsevier Science Ltd.

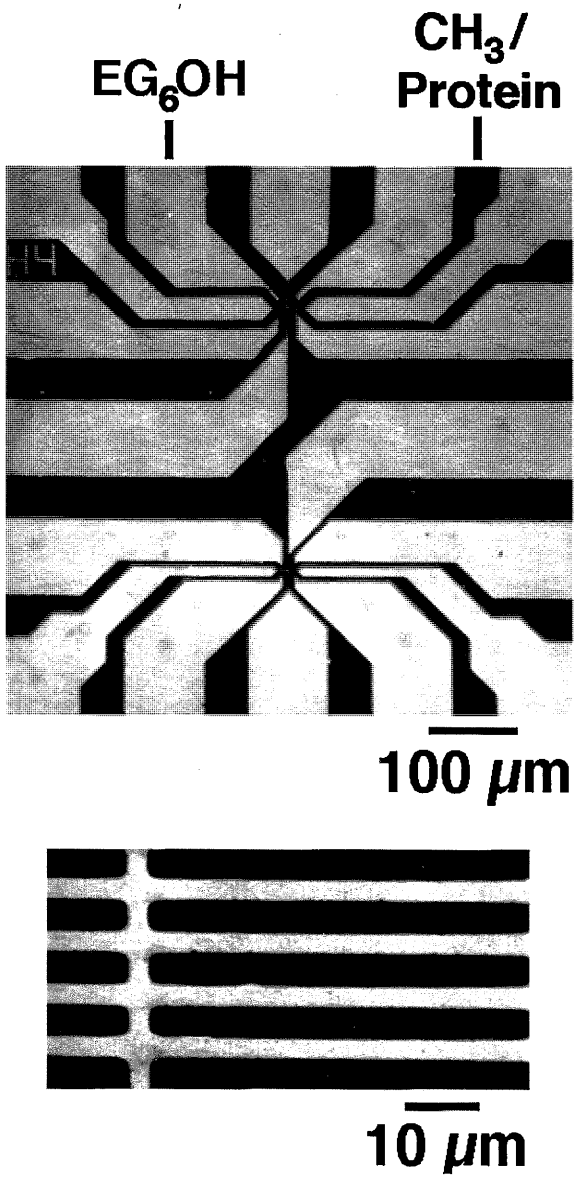

Fig. 5

methyl groups (hydrophobic groups) or oligo(ethylene glycol) groups. Immersion of these SAMs in an aqueous solution of protein resulted in the adsorption of a monolayer of protein on the methyl-terminated regions; scanning electron microscopy (SEM) is a particularly convenient technique for imaging the resulting pattern of protein (Fig. 5).[59] Bhatia et al used photolithography to convert siloxane SAMs terminated with thiol groups to sulfonate groups which resisted the non-specific adsorption of protein; the fluorescent protein phycoerythrin was then covalently linked to the thiol groups in regions that had been protected from UV light by the mask.[60]

Cell Attachment to Patterned SAMs. The same methods used to create patterns of proteins have been used to control the attachment of cells to surfaces:[61-64] Spargo et al used photolithography to prepare patterned arrays of perfluoro-and amino-terminated siloxane monolayers; endothelial cells preferentially adhered to fibronectin-covered regions of the amino-terminated SAMs and differentiated into neovascular cords.[62] Amino-terminated siloxanes are excellent substrates for culture of neural cells.[63]

Microcontact printing was used to pattern SAMs in adhesive lines ranging from 10 μm to 100 μm in width; after coating these substrates with fibronectin, endothelial cells attached and grew only on those lines (Fig. 6). Microcontact printing was similarly used to form SAMs containing adhesive islands (as small as 1600 μm^2) surrounded by oligo(ethylene glycol)-terminated SAMs.[64] Hepatocytes attached to the laminin-coated hydrophobic islands. DNA synthesis, cell growth, and albumin secretion of the attached hepatocytes was found to be a function of the island size. The use of SAMs to confine growth of cells to specific areas and shapes should increase understanding of the relationship between cell shape and function. The ability to pattern the attachment of individual cells may also be useful for single cell manipulation, toxicology, and drug screening assays.

Figure 6. Controlled attachment of bovine capillary endothelial cells to planar substrates patterned into regions of SAMs terminated in methyl groups and tri(ethylene glycol) groups using microcontact printing. The substrates were coated with fibronectin prior to cell attachment; fibronectin adsorbed only to the methyl-terminated regions of the SAM. (A) The optical micrograph shows attachment of endothelial cells to the non-patterned region at left and to lines 30 μm in width. (B) A view at higher magnification of cells attached to the lines.

Cell Attachment on Contoured Surfaces. Several groups have used surfaces contoured in grooves and ridges--fabricated using photo- or electron beam lithography--to study surface topography on the growth and behavior of attached cells.[65-67] Chou and coworkers found that human fibroblasts adherent to surfaces contoured into V-shaped grooves had increased levels of fibronectin synthesis and secretion relative to those grown on smooth surfaces.[65] Similarly grooved substrata facilitated the *in vitro* healing of flexor tendons relative to planar substrata.[66] The dimensions of ridges on silicon wafers patterned with arrays of ridges was found to signal differentiation for the fungus *Uromyces.*[67]

Electrochemical Control Over Interfacial Properties. The electrical conductivity of the gold supporting a SAM of alkanethiolates permits a variety of strategies to control the properties of the interface. The wetting properties of SAMs terminated in electroactive groups--for example, ferrocene and quinone groups--were switched reversibly by applying reducing and oxidizing potentials to the gold electrode.[68] Several groups have immobilized electroactive proteins to SAMs on gold to study electron-transfer reactions of the proteins.[69] Wong, Langer, and Ingber have shown that the shape and growth of aortic endothelial cells adherent to conducting films of fibronectin-coated polypyrrole were controlled by applying a potential to the substrate.[70]

Prospects. SAMs of alkanethiolates on gold constitute a convenient and broadly useful model system for studying protein adsorption--and processes dependent on protein adsorption--at interfaces. The ability to control precisely the surface chemistry through synthesis, to pattern the formation of SAMs using μCP, to control the optical and electrical properties of the interface, to employ a variety of analytical techniques that are compatible with SAMs, and to use a variety of substrates--including non-planar and

contoured substrates--permits a wide range of flexibility in tailoring this system for specific applications. A few early demonstrations are described in this chapter; many more will certainly follow.

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