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The interaction of proteins and cells with self-assembled monolayers of alkanethiolates on gold and silver

Emanuele Ostuni, Lin Yan, George M. Whitesides *

Department of Chemistry and Chemical Biology, Harvard University, 12 Oxford Street, Cambridge, MA 02138, USA

Abstract

Alkanethiols, $HS(CH_2)_nX$, chemisorb on gold and silver and form self-assembled monolayers (SAMs). The ability to present a variety of functional groups, X, at the terminal position of the alkanethiol makes it possible to control the structure of the surface at the molecular level, and thus to control the interfacial properties of these organic surfaces. These SAMs constitute an exceptionally useful set of model surfaces with which to study the interaction of synthetic materials with biologically relevant systems. By varying the terminal group X, it is possible to examine the influence of the structure and polarity of common organic groups on the adsorption of proteins. Alkanethiols terminated with oligo(ethylene glycol) groups form SAMs that resist the adsorption of proteins (so-called 'inert surfaces'). These alkanethiols, when used in mixed SAMs that include alkanethiols that present other functional groups, isolate the biomolecular interactions of interest from non-specific effects and simplify fundamental studies of protein adsorption. Surface plasmon resonance (SPR) is a particularly valuable technique for measuring rates and equilibrium constants of processes that involve adsorption of proteins at surfaces and for characterizing mechanisms of protein adsorption. Since the techniques used in preparing SAMs for studies of protein adsorption are essentially the same as those used in preparing substrates for SPR, a common synthetic technology can be used with both. Soft lithographic techniques—microprinting and micromolding—make it possible to pattern SAMs with different functionalities on surfaces that can be either planar or contoured. The combination of SAMs, inert surfaces, SPR, and soft lithography allows the study of the molecular-level interaction of solutions containing proteins with synthetic surfaces. Extensions of these studies to investigations of the attachment and spreading of cells on surfaces also offer a new set of research tools in cell biology.

Keywords: Alkanethiols; Adsorption; Soft lithographic techniques; Synthetic surfaces

1. Introduction

1.1. Objective and scope of the review

This review focuses on the value of self-assembled monolayers (SAMs) of alkanethiolates on gold and silver as model organic surfaces [1,2], and on the use of these systems to characterize (and in some cases to control) the interactions of important types of biomolecules (especially proteins) with surfaces [3,4]. Patterning SAMs on gold using soft lithographic techniques [5–7] affords spatial control over the adsorption of proteins [8–10] and makes it possible to study the

* Corresponding author. Tel.: +1-617-4959430; fax: +1-617-4959857.

E-mail address: gwhitesides@gmwgroup.harvard.edu (G.M. Whitesides)

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interaction of cells [11–14] with surfaces that present features of different sizes, shapes, and topologies.

1.2. The interaction of synthetic materials with biological systems: proteins and cells

Controlling the interaction of biomolecules and cells with the surfaces of man-made materials (which we will call 'synthetic' materials) is broadly important in biochemistry, biology, biotechnology, and health care. Table 1 lists some representative applications of synthetic materials in these fields. Proper functioning of most biomedical devices requires proper functioning of their surfaces. The application of rational principles of interfacial chemistry to the design and fabrication of the surfaces is important in improving the performance of devices of which they are a part, and in designing and developing new biomaterials with specific functions.

Table 1
Materials used in medicine and biotechnology [187–192]

Material	Application
Pyrolitic carbon	Heart valves
Platinum electrodes	Cochlear replacement
Titanium; alumina; calcium phosphate	Dental implants
Titanium alloy	Joint replacement
Nitinol	Vascular stents
Hydroxylapatite	Bony defect repair
Poly(urethane)	Artificial heart
Dacron, teflon	Artificial tendon and ligament
	Vascular prosthesis
Poly(dimethylsiloxane) (PDMS)	Breast, chin, and nose implants
	Blood vessels; heart valves
Poly(ethylene)	Hip replacements
Poly(hydroxyethyl methacrylate)	Soft contact lenses
Cyanoacrylate	Surgical adhesive
Silicon–collagen composite	Skin repair template
Polypropylene, nylon	Unresorbable sutures
Poly(methyl methacrylate)	Contact lenses
	Bone cement
Poly(glycolic acid)	Bioresorbable suture, stent, pin,
and poly(lactic acid)	Artificial tendons and ligaments
Polyethylene terephthalate	Leaflet valves: arteries and veins
Poly(styrene)	Tissue culture

When a synthetic material is brought into contact with a biological fluid, the first event that occurs is usually the adsorption of proteins onto its surface; the protein that adsorbs to the surface first may be replaced subsequently by others that adsorb more tightly [15]. Many aspects of the subsequent performance of the material are influenced by the layer(s) of adsorbed protein(s). The mechanisms of these adsorption events have been studied and reviewed extensively, but the subject is a difficult one technically and many aspects of the mechanisms of the adsorption of proteins remain speculative [15]. The broad assumption in discussions of these interactions is that the initial stages of many processes ending in non-biospecific adsorption involve hydrophobic interactions between the surfaces of the material and hydrophobic patches on the surface of the protein [15,16]. Following an initial adsorption that is probably weak and reversible, the protein may denature and unfold to expose more hydrophobic groups, and ultimately attach irreversibly to the surface. For a solution containing multiple proteins or other biomolecules, the complexities of time-dependent adsorption processes [16,17] have made a complete description of the problem at the molecular level intractably difficult.

The ability to control the interaction of proteins with surfaces at the molecular level using SAMs is one component of a set of research tools for designing and fabricating materials that direct the attachment and biological response of mammalian cells. The interaction of cells with surfaces can be organized conceptually into three stages. On exposure of the synthetic material to the suspension of cells in culture medium, the first stage is adsorption of proteins from the medium to the surface [15]. The second stage—attachment and spreading of cells at the surface—depends on the interaction of the cells with the adsorbed proteins. The third stage involves modification of the composition of the

interfacial layer by proteins and carbohydrates produced by the cell (also called 'surface remodeling' [18]. There are exceptions to this paradigm, [19] and it is undoubtedly oversimplified, but it is useful in organizing the analysis of the subject.

2. SAMs, 'inert surfaces', soft lithography, and SPR

Four techniques have emerged in the last decade that have proved synergistic, and that have made it possible to carry out molecular-level studies of the interaction of biomolecules with synthetic surfaces. SAMs (especially SAMs of alkanethiolates on gold and silver) provide practical systems around which to design and synthesize surfaces [20–22]. Using these systems it is, for the first time, relatively straightforward to tailor both the physical and chemical properties of biologically relevant interfaces [4]. The ease with which simple alkanethiols can be synthesized, and the compatibility of these synthetic methods with biochemical functional groups, makes it straightforward to present even structurally complex groups at the surface of SAMs (Fig. 1) [19,23].

For a number of types of experiments, it is helpful or even essential to make surfaces that resist the adsorption of proteins—surfaces that we call 'inert' (meaning 'inert to the adsorption of proteins'). The unavailability of inert surfaces has, in the past, made research in biosurface chemistry troublesome and ambiguous: the adsorption event of interest (whether biospecific or not) always had to be recognized against a strong background of competing non-specific adsorption [24–27]. The recognition that SAMs that present high densities of oligo(ethylene glycol) moieties effectively resist the adsorption of proteins makes it possible to reduce this background significantly—often to negligible levels—and to study different types of adsorption events, both physical (non-biospecific) and biospecific, in isolation (Fig. 1) [8,23,28–30]. This capability to reduce non-specific background adsorption enormously increases the significance of the information obtained from experiments. Since the analytical techniques—surface plasmon resonance (SPR) spectroscopy, [31–35] X-ray photoelectron spectroscopy (XPS) [36], quartz crystal microbalance (QCM), [37,38] etc.—used to characterize surfaces in these environments are relatively information-poor and cannot discriminate among several interactions (and often even among different proteins), it is important to design and perform the simplest experiments possible. The key to such studies is the formation of mixed SAMs that isolate the interaction of interest, and eliminate, as completely as possible, other competing interactions. Alkanethiols that present oligo(ethyleneglycol) ($EG_{n=3-6}$) groups form SAMs that resist the adsorption of proteins; they can be used to form mixed SAMs through coadsorption with alkanethiols that present $(EG)_6OR$ groups—where R is the ligand of interest [23,39]. The portion of the EG_6OR moiety that protrudes above the protein-resistant background formed by the EG_3OH groups is also protein-resistant and does not introduce undesired non-specific effects in the experiment (Fig. 1) [23,40]. We have used this system successfully with a number of ligands (Fig. 1) for the study of bio- and non biospecific interactions at the surfaces of SAMs [23,40,41].

SAMs provide the capability to engineer the surface with Ångstrom-level detail perpendicular to the mean plane of the surface. In many experiments—especially when working with attached cells—patterning *in* the plane of the surface is also important. A new set of techniques (collectively called 'soft lithography' for their reliance on an elastomeric stamp or mold to transfer the pattern to the surface [7]) has now provided this capability. Although the resolution of soft lithographic patterning is limited to features greater than a few hundred nm [4,7,42–44], this feature size is entirely adequate for most types of experiments with cells (which are entities with dimensions of tens of microns). The patterned surfaces direct the adsorption of proteins and hence direct cellular adhesion [11,13,14].

The last technique that combines synergistically to complete the quartet of techniques is surface plasmon resonance spectroscopy (SPR) [31]. SPR can give useful information about the rates of the processes leading to adsorption and desorption.

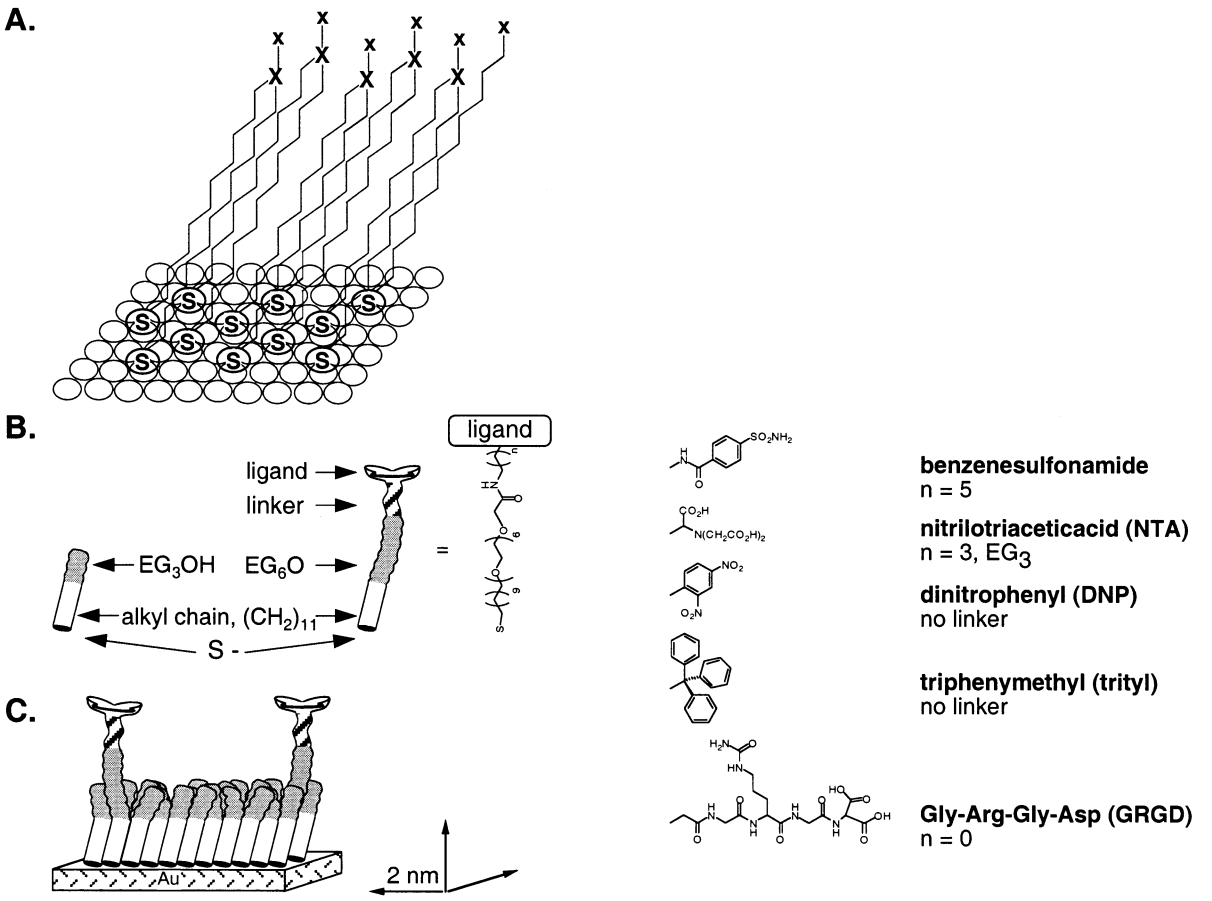


Fig. 1. (A) Schematic diagram of a self-assembled monolayer of an alkanethiolate on gold. The polymethylene chains are oriented 30° from the surface normal and pack with nearly crystalline densities; the interfacial properties of the film are largely determined by the chemical properties of the terminal group X. The details of the structure of the SAM–Au interface are largely irrelevant to processes that occur at the SAM–solution interface (see text). (B) Model of an alkanethiol that presents a ligand. The structures of several ligands that we have presented at the surface of SAMs are shown. Typically, we present ligands at a surface using undecylenic thiols that are terminated with an hexa(ethylene glycol) ($(EG)_6$) group; the linker is the moiety that connects the terminal oxygen of the $(EG)_6$ functionality to the ligand of choice. (C) Effective ligand display is obtained by mixing alkanethiols that present $(EG)_3OH$ and $(EG)_6OR$ groups, where R is the ligand of interest. This schematic drawing illustrates mixed SAMs prepared from these alkanethiols (prepared roughly to scale, but with no implication concerning the detailed structure of the surface layer): the trityl ligand protrudes above the $(EG)_3OH$ layer that is resistant to the adsorption of proteins (see text).

SPR *requires* a thin, semitransparent film of metal (typically gold, although silver has also been used), and is thus ideally complementary to, and capable of taking advantage of, SAMs of alkanethiolates on gold (Fig. 2). This technique provides information on the amount of protein that adsorbs to the surface, but it gives no details on the molecular structure of the adsorbed protein.

The combination of these four techniques now enables the study of phenomena occurring at the interface between synthetic materials and biological systems with a detail and convenience that has not been possible in the past.

2.1. SAMs as molecularly defined organic surfaces: alkanethiolates on gold

2.1.1. Types of SAMs

Alkanethiols chemisorb, from solution or vapor, onto surfaces of Au, Ag, and Cu and form

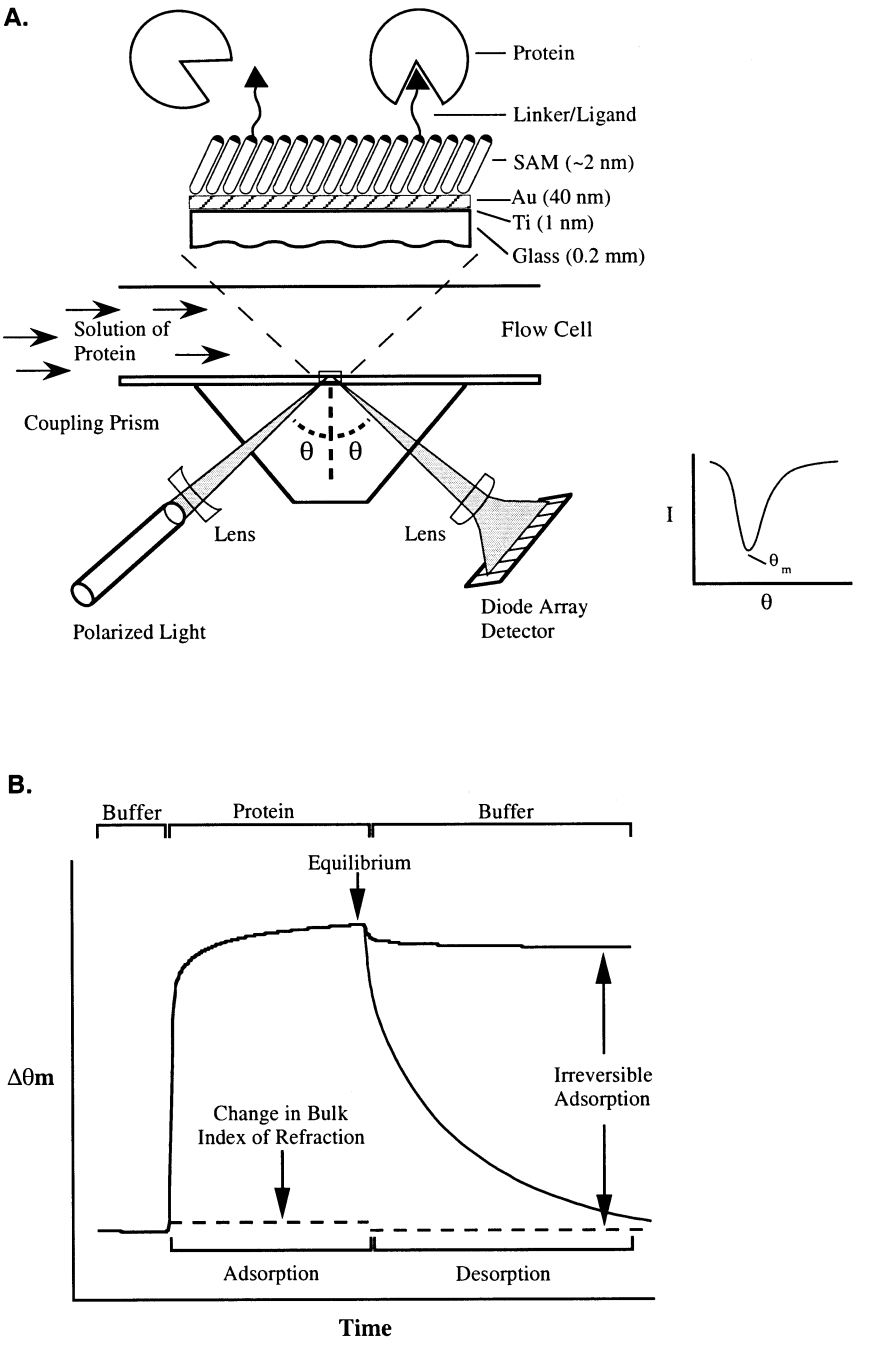


Fig. 2. (*Continued*)

SAMs [2,20,21,45–47]. The nature of the gold–sulfur bond is still a subject of discussion [48–50]; we adopt the view that the species present at the surface is a gold(I) thiolate. The mechanisms of these reactions have not been established—Eq. (1) applies only to Au—but it is known that the

$RS^- - Au$ bond is relatively stable, with $\Delta H° \approx$ 28 kcal/mol [51,52]. The surface of

$$X - R - SH + Au(0)_n \rightarrow X - R - S^- Au(I) \cdot Au(0)_{n-1} + \frac{1}{2}H_2 \quad (1)$$

gold is more resistant to oxidation than most other metals, and it is the one we have chosen to use as the substrate for model studies of the interaction of biological media with interfaces defined by SAMs. SAMs of alkanethiolates on Ag are structurally more ordered than on Au [53], but they suffer from the defect that they seem to release Ag(I) ion into solution: this ion is cytotoxic, and makes it necessary to work with SAMs on gold for studies of cells. Gold substrates covered with SAMs are not toxic to living cells and withstand the conditions used in cell culture; they can be used to pattern the adhesion of cells and study related phenomena. These SAMs are reasonably stable: alkanethiolates desorb from the surface of gold when irradiated with UV light in an oxygen atmosphere [54–56], when exposed to atmospheric ozone [57,58], when heated above 70°C [59], or when exposed to kiloelectronvolt ion bombardment [60].

2.1.2. Preparation of SAMs of alkanethiolates on gold

The preparation of SAMs on gold is straightforward. The substrates are prepared on glass cover slips or silicon wafers by evaporating a thin layer of titanium (1–5 nm) to promote the adhesion of gold, followed by a thin film of gold. We find that a layer of gold as thin as 12 nm is sufficient for the formation of continuous, well-structured SAMs; these gold substrates are only partially reflective and make it possible to view the adhesion of cells with an optical microscope [61]. We usually use 38 nm thick layers of gold for SPR; a layer of this thickness is sufficient to allow the total internal reflection of the laser beam used in SPR. The gold-coated substrates are cut to the dimensions required by the application and immersed in a solution (typically ethanolic, 2–10 mM) of the alkanethiol(s) for several hours followed by rinsing with the same solvent and drying with nitrogen [4,62,63]. Although complete SAMs of a single alkanethiol on gold form in < 2 min, we usually choose to leave gold substrates in solutions of *mixtures* of alkanethiols for 8 h [23,41]. This choice is, to an extent, arbitrary.

Alkanethiols that present functional groups recognized specifically by proteins can often be synthesized according to the procedures outlined in Eq. (2) [8,19,23,28,40]. The attachment of complex carbohydrates and polypeptides to alkanethiols may require the use of protecting groups [19]. Usually, the terminal hydroxyl group of the $(EG)_n$ moiety is used as a nucleophile for the S_N2 displacement of a bromide from the ligand. Radical addition of thiolacetic acid across the

Fig. 2. Surface plasmon resonance permits the measurement of the adsorption of proteins at surfaces in situ. (A) A beam of p-polarized light is used to illuminate the back side of a thin film of gold supported on glass and in contact with a solution of interest. At a specific angle θ_m, the light excites plasmons (collective excitations) of the electrons at the surface of the gold: this excitation removes energy from the beam and is detected as a decrease in the intensity of the reflected light. The values of θ_m change ($\Delta\theta_m$) as the index of refraction of the interface changes (e.g. by the adsorption of protein) as a function of time. This technique can be used to measure the quantity of protein adsorbed at an interface in real time, and thus provide information about the rates and equilibria of adsorption. (B) In a typical SPR sensorgram, $\Delta\theta_m$ is plotted as a function of time. A solution of buffer flows (at 5 μl/min) for 2 min over a cell in contact with the SAM, followed by 3 min of buffer containing protein and four additional minutes of buffer (to wash the surface). The relevant regions of the sensorgram are labeled. We show shapes of sensorgrams that are typical of reversible and irreversible adsorption processes. The dashed line illustrates the bulk change in the index of refraction of the solution that is detected when buffer is exchanged with buffer containing protein. When recording a sensorgram, the instrument detects this bulk change before the change that is associated with the adsorption of a protein at the interface.

$$H_2C=CH(CH_2)_9(OCH_2CH_2)_nOH \xrightarrow[3.\ NaOCH_3/CH_3OH]{1.\ NaOH;\ RBr \quad 2.\ CH_3COSH/h\nu} HS(CH_2)_{11}(OCH_2CH_2)_nOR \qquad (2)$$

double bond (78–88% yield), followed by basic hydrolysis of the thioacetate, afford the thiols in good (84–91%) yields [28].

It is often more convenient to prepare surfaces that present complex ligands by modifying the terminal groups of alkanethiolates *after* their assembly into SAMs [64–67]. Treatment of a SAM that presents carboxylic acid groups with trifluoroacetic anhydride forms inter-chain anhydrides; this reactive group can be converted easily to an equimolar mixture of amides and carboxylic acids through a nucleophilic reaction with an alkylamine that bears the functional group of interest, R (Fig. 3 A). An alternative method for the formation of mixed SAMs that present EG_3OH and EG_6OR groups is described in Fig. 3B [66]. Mixed SAMs are formed from mixtures of EG_3OH and EG_6OCH_2COOH groups. The acid groups are functionalized with an *N*-hydroxysuccinimide (NHS) group that is easily displaced by amines that bear the ligand of interest. This approach is convenient for controlling the density of complex ligands in a SAM when the synthesis of the corresponding alkanethiol would otherwise require the use of multiple protecting groups.

Other methods for the derivatization of SAMs have also been developed. Amines can react with SAMs that present aldehyde groups to form imines [68] and with acid chloride groups [69] to form amides. Terminal trifluoromethyl esters can be synthesized by the reaction of trifluoroacetic anhydride with SAMs that present an alcohol group [70,71]. Electrochemistry can also be used to *release* terminal groups into solution by cleavage of the linking groups from SAMs [72].

2.1.3. Structures and characterization of SAMs of alkanethiolates on gold

The structure and the properties of SAMs have been studied extensively because these surfaces provide good model systems for fundamental studies of tribology, adhesion, wetting, corrosion, biosurfaces, and cell biology [2,4,45,46,73–75]. The packing density of alkanethiolates on gold is influenced by the spacing of the co-ordination sites [76]. Diffraction studies suggest that the sulfur atoms are localized in three-fold hollow sites of the Au (111) surface [77,78]—the structure of the metal surface that normally predominates in films formed by thermal evaporation. Fenter et al. have interpreted recent X-ray diffraction studies to indicate that alkanethiols chemisorb onto the gold as disulfides [48,49]. The structure of the alkanethiolate layer seems to require an average projected area of 21.4 $Å^2$/sulfur atom, while the cross-sectional area of the alkane chain is 18.4 $Å^2$ [53]. This difference in density requires that the alkyl chains tilt by 30° with respect to the surface normal of the gold in order to minimize free volume and maximize van der Waals interactions between polymethylene chains (Fig. 1A) [53]. The chemical properties of the head group (X) do not strongly affect the structure of SAMs of $HS(CH_2)_nX$ on gold, if the size of X approximates that of a methyl group or is smaller. Nuzzo and co-workers have shown that for values of $n \geq 10$ the molecules adopt a predominantly trans conformation and that the number of gauche bonds decreases as n increases [75,79]. As a result of van der Waals interactions between adjacent polymethylene chains, SAMs of alkanethiolates on gold pack at densities that approach those of crystalline polyethylene; this semi-crystalline layer is relatively impermeable to molecules in solution [63]. Studies of biosurfaces generally involve ligands with biological activity that are significantly larger than a methyl group; no detailed structural studies are, however, available for the surface layer of SAMs presenting large ligands.

In SAMs on both silver and copper, the angle of tilt of the polymethylene chains is ca. 13° [53]. The distance between nearest neighbors in the (111) plane on silver (2.89 Å) is similar to that of gold (2.88 Å) and different from that of copper (2.56 Å); these structures differ significantly from that of SAMs on gold. Prolonged exposure of the metal films to ambient air alters the structure of the surfaces of silver and copper and those of the resulting SAMs. SAMs formed on carefully prepared silver and copper substrates generally have

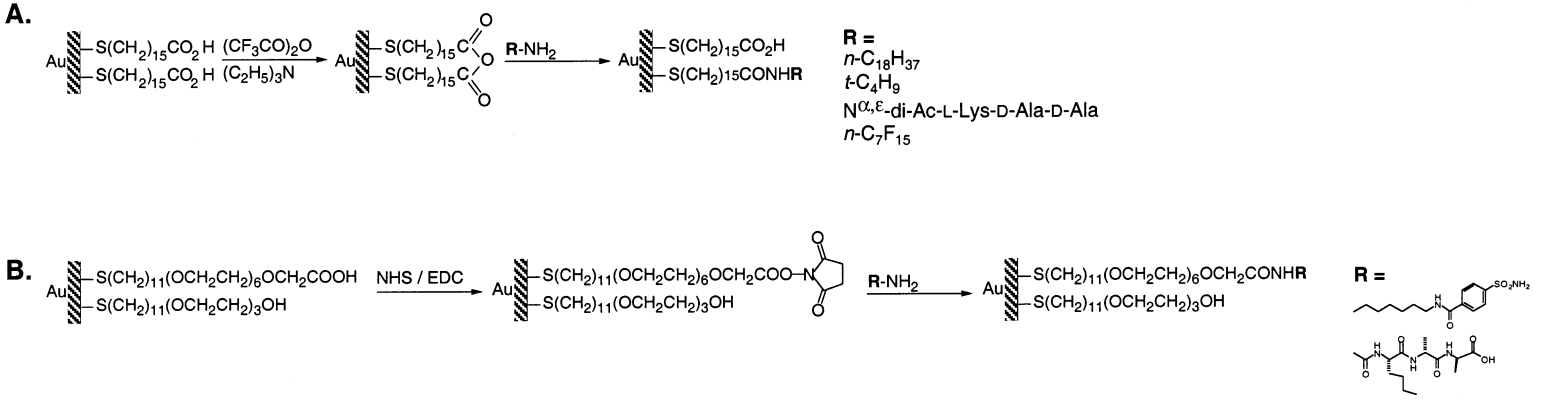


Fig. 3. Mixed SAMs can be prepared by performing reactions *on* pre-formed SAMs. (A) Alkylamines (in 1-methyl-2-pyrrolidinone) react with interchain carboxylic anhydride groups formed on SAMs of $HS(CH_2)_{15}COOH$ after reaction with trifluoroacetic anhydride and triethylamine in anhydrous *N*, *N*-dimethylformamide [64]. This procedure generates a surface that presents an equimolar mixture of the ligand R and carboxylic acid groups on the alkanethiolate molecules of the SAM. The structures of representative R groups are shown. (B) An alternative method for the formation of mixed SAMs that present complex ligands at a surface that does not otherwise adsorb proteins. SAMs are formed that comprise a mixture of carboxylic acid-capped hexa(ethyleneglycol)- and tri(ethyleneglycol)-terminated undecanethiolate. *N*-hydroxysuccinimide (NHS) and *N*-ethyl-*N*′-(dimethylaminopropyl)carbodiimide (EDC) then react with carboxylic acid groups on this surface to form an active ester; this active ester subsequently reacts with alkylamines that carry the ligand of interest, R [66]. The structures of two R groups that have been immobilized with this technique are shown.

a lower population of gauche bonds than SAMs on gold [53].

2.1.4. SAMs of alkanethiolates on gold as model systems for physical–organic studies of surfaces

The properties of SAMs make them excellent models for physical–organic studies of the solid–liquid interface. We and others have formed SAMs from mixtures of alkanethiols to study the effect of interfacial molecular structure on the wettability of an organic surface (Table 2) [2,80,81]. A factor that might affect the surface properties of mixed SAMs is phase segregation of the two alkanethiols. A number of different mixed systems have been studied [8,28,39,63,74,82,83] and to date, it is not possible to make general statements on the extent of phase segregation in mixed SAMs. Each system must be dealt with separately; the results depend on the structures of the alkanethiols and the details of the process of self-assembly.

The composition of mixed SAMs can be substantially different from that of the mixtures of alkanethiols in the solutions used to prepare them. For this reason, it is advisable to measure the surface composition of mixed SAMs *after* their assembly. XPS is particularly well-suited to measure the surface compositions of SAMs formed from alkanethiols that contain different types of atoms [23,81,84]. At low mole fractions of a ligand (typically $\chi < 0.1$), the sensitivity of XPS is, however, poor and surface compositions of SAMs cannot be determined accurately. Ellipsometric and contact angle measurements complement XPS in the characterization of SAMs [39,63]. The properties of the solvent used during the coadsorption also influence the surface composition of the SAM [39]. When using mixed SAMs to tailor interfacial properties of materials, one must consider the structures of the alkanethiols in detail. We generally present the ligand of interest with an alkanethiol that is longer than that used for the remainder of the SAM so that the ligand is accessible to proteins (Fig. 1C). In such cases, the wettability and the ability to bind other molecules of the SAM will be affected not only by the head group but also by the properties of the exposed alkyl chain [82].

Table 2
Advancing contact angles (θ_a) of water on SAMs of $^{-}S(CH_2)_nX$ on gold

n	X	θ_a(°)
2	$(CF_2)_5CF_3$	119 [53]
11	$OCH_2CF_2CF_3$	118 [53]
16	$O(CH_2)_4CH_3$	115 [63]
16	$O(CH_2)_3CH_3$	113 [63]
15	CH_3	110 [75]
16	$O(CH_2)_2CH_3$	110 [63]
17	$CH=CH_2$	105 [53]
11	$O(CH_2)_2CH_3$	104 [63]
11	OCH_2CH_3	96 [63]
15	CO_2CH_3	95 [75]
11	$OCOCF_3$	93 [53]
10	$CO_2CH_2CH_3$	89 [53]
11	Cl	89 [53]
11	OCH_3	85 [53]
0	$(p\text{-}C_6H_4)(p\text{-}C_6H_4)CH_3$	84 [193]
8	CH_3	82 [63]
11	$CONHCH_3$	76 [53]
12	$SCOCH_3$	70 [194]
16	CN	70 [53]
16	$OCO(C_6H_5)$	68 [195]
16	$OCOCH_3$	64 [95]
11	$(OCH_2CH_2)_3OCH_3$	40 [8]
11	$(OCH_2CH_2)_5OH$	38 [28]
11	$(OCH_2CH_2)_6OH$	38 [28]
11	$(OCH_2CH_2)_3OH$	34 [28]
0	C_6H_4-4-(C_6H_4-4-OH)	33 [193]
11	OH	<15 [63]
15	CO_2H	0-10 [75]
15	CH_2OH	0-10 [75]
15	$CONH_2$	0-10 [75]

2.2. The 'inert surface' problem and SAMs presenting $(EG)_nOH$ groups

SAMs formed with an undecylenic thiol bearing oligo(ethylene glycol), EG_nOH, groups with $n = 2-7$ resist the adsorption of proteins with a range of molecular weights, pI's, and net charges (Fig. 4) [29,85]. This result is not obviously compatible with the theoretical predictions of Andrade and De Gennes that poly(ethylene glycol) (PEG) chains grafted on the surface of a polymer would become less resistant to the adsorption of proteins as they become shorter [86,87]. In a recent experimental study of the protein resistance of PEG grafted by irradiation onto glass modified

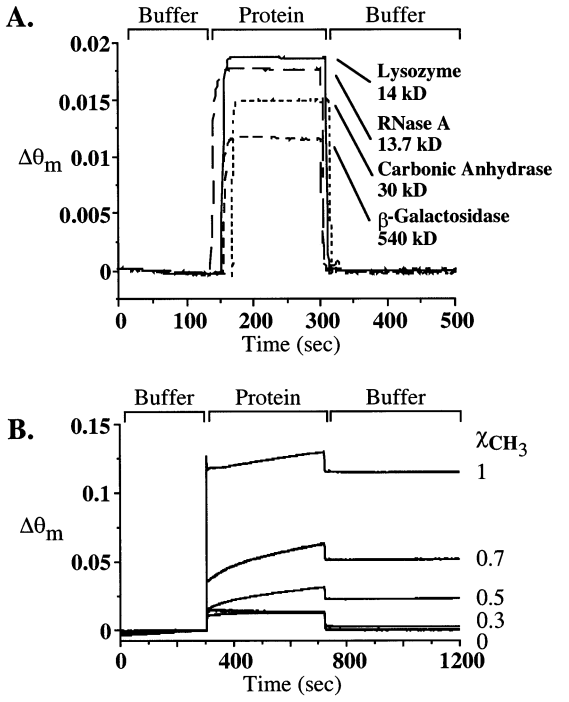


Fig. 4. SAMs presenting tri(ethylene glycol) groups resist the adsorption of proteins. (A) The SPR sensorgrams show that insignificant amounts of proteins adsorbed to the SAM; after the flow of the solution of protein over the SAM was switched back to buffer, the signal returned to the baseline obtained prior to the injection of protein. The shape of the sensorgram is typical of a bulk change in index of refraction (see Fig. 2) rather than of the adsorption of proteins. (B) The formation of mixed SAMs comprising oligo(ethylene glycol) and methyl groups can be used to vary the extent of protein adsorption at a surface. The amount of protein that adsorbs to these mixed SAMs decreases when the mole fraction (χ_{CH_3}) of methyl groups decreases and the mole fraction of protein resistant groups increases [41]. Upon removing the protein from the buffer flowing over the surface, the signal does not return to the level obtained before the injection of protein. The protein adsorbs onto these surfaces irreversibly.

with trichlorovinylsilane, McPherson et al. [88] confirmed, partially, the predictions made by Andrade and co-workers. The calculations of Andrade and Jeon and the theory of De Gennes suggest that PEG chains (typically with 80–120 monomers) attached to polymers are hydrated and that water molecules are compressed out of the PEG chains as the protein approaches the surface [86,87]. Andrade and Jeon believe that the 'steric repulsion' generated by this thermodynamically unfavorable interaction is responsible for the inertness of the PEG surface [86,87].

SAMs that present oligo(ethylene glycol) groups seem to resist the adsorption of proteins via a mechanism that is different (at least in its details) from that suggested by Andrade and Jeon. The density of $(EG)_nOH$ achieved in a SAM is higher than that used in the calculations of Andrade; Chin et al performed molecular dynamics simulations that suggest that the interior of the $(EG)_n$ layer of the SAM is not hydrated [89]. Grunze and co-workers used IR spectroscopy to identify loosely bound water molecules *at the surface* of a SAM terminated with an $(EG)_3OH$ group [90]. These workers have reported a suggestive correlation: $(EG)_nOH$ moieties that, when dry, adopted a helical conformation in the SAM were protein-resistant when in contact with water; similarly, SAMs in which the $(EG)_nOH$ groups were either all trans (that is, crystalline) or were highly disordered allowed protein adsorption [91].

$(EG)_nOH$ groups are effective in resisting the adsorption of proteins, and PEG chains have been used routinely to improve the solubility and the biocompatibility of molecules and materials used in the pharmaceutical industry [92]. $(EG)_nOH$ groups, however, oxidize over time and are not ideal for long-term applications in vivo—for example, in artificial organs. In prospecting for other groups that are protein-resistant, we have demonstrated that $(EG)_nOH$ groups are not unique in their ability to resist the adsorption of proteins: SAMs presenting tri(propylenesulfoxide) groups $[CH_2CH_2CH_2S(O)]_3$ also resist the adsorption of protein [93]. Chapman et al. found that groups that resist the adsorption of proteins are hydrogen bond acceptors but not hydrogen bond donors, hydrophilic, and conformationally flexible [94].

2.3. Analytical systems for studying the interactions of biological molecules with surfaces

The ideal analytical method for studying the interaction of complex biological media with surfaces should: (i) allow the detection of the interaction in situ; (ii) provide kinetic and thermodynamic information about the process;

(iii) avoid the need for covalent modification of the analyte; (iv) use small amounts of material; (v) have a high signal-to-noise ratio that allows the detection of sub-monolayer quantities of material; and (vi) have high specificity for individual structures. No single technique meets all of these criteria [17].

2.3.1. Surface plasmon resonance (SPR)

Surface plasmon resonance (SPR) is sensitive to changes in the index of refraction at and near the surface of a metal film (Fig. 2) [31–35,95]. SPR is an in situ technique for the observation of processes occurring between synthetic surfaces and complex biological solutions that allows the acquisition of data in real time without requiring fluorescent tagging of the analytes, and is convenient for obtaining both kinetic and thermodynamic parameters. SPR can detect conformational changes of biomolecules immobilized at a surface [96] only if they lead to changes in the index of refraction of the layer. SPR instruments are commercially available from BIAcore, Affinity Sensors, and Texas Instruments; they can also be custom-built [97,98]. Although the optical detection units can be assembled relatively easily, these customized instruments often lack precise fluidic handling and require relatively large volumes of sample (5–10 ml). The Biacore SPR spectrometer [95,99,100] is the only one of these three instruments that both: (i) uses exchangeable cartridges containing planar gold substrates that lend themselves to derivatization with SAMs; and (ii) requires low volumes (10–50 μl) of analyte solution. The configuration of the BIAcore instrument available in 1998 does not allow the measurement of the adsorption of molecules with a molecular weight lower than ≈ 200 g/mol.

The use of SAMs to immobilize ligands to a surface and to study the adsorption of biomolecules allows more controllable SPR measurements of binding events at these surfaces than does the use of the dextran layer available from BIAcore. The intensity of the evanescent wave that is sensitive to the index of refraction of the interfacial layer of fluid decays exponentially with distance from the surface. Chips commercially available from Biacore use a gold substrate derivatized with a relatively thick (ca. 100 nm) dextran matrix that can be functionalized to effect immobilization of ligands. The properties of the matrix impose several limitations on this process [34,40,101,102]: (i) it is thick enough to cause kinetic and thermodynamic partitioning of the analyte in the gel matrix and it can influence the thermodynamic and kinetic parameters of adsorption; (ii) it allows the immobilization of ligands at densities high enough to introduce multivalent effects in the adsorption events; and (iii) its residual negative charge after derivatization may cause the nonspecific adsorption of some positively charged proteins.

2.3.2. Ellipsometry [103]

Ellipsometric measurements of protein adsorption can be performed in situ, although there are no commercially available instruments capable of reliably collecting data that are suitable for kinetic analysis of processes as fast as the adsorption of protein [104]. Mrksich et al. demonstrated a direct correlation between the ellipsometric thickness of protein layers ex situ and the adsorption measured by SPR on mixed SAMs in situ [41].

2.3.3. Quartz crystal microbalance (QCM)

QCM is a sensitive ex situ technique that has recently been adapted to in situ measurements [37,38]. QCM uses the piezoelectric effect to measure changes in the fundamental frequency of vibration of a quartz crystal as protein adsorbs to it: these changes are proportional to the thickness of the adsorbed layer of material. The operating frequency of the instrument is affected by the presence of bulk liquid at the interface; this change is different than and can be subtracted from the signal generated by the adsorbed molecules [37]. QCM measurements can be performed on a variety of substrates. Quartz substrates with polymeric coatings have poor substrate specificity. More recently, QCM measurements have been performed on gold substrates functionalized with SAMs [105–107].

2.3.4. Surface acoustic wave (SAW) and acoustic plate mode (APM) devices [37,108]

SAW and APM devices detect changes in the amplitude and velocity of surface acoustic waves (launched by interdigitated electrodes on the surface of a suitable crystal) resulting from the adsorption of a biomolecule onto the surface [109]. In these techniques, detection is sensitive to turbulence in the liquid medium; the unstirred systems may require equilibration times that are too long (in the order of hours) for practical detection schemes. The mass of the liquid at the interface is large compared to that of the protein and it decreases the sensitivity of the measurement. Surface derivatization is usually performed with alkylsiloxane chemistry; these methods are not as convenient as those developed for SAMs of alkanethiolates on gold.

2.3.5. X-ray photoelectron spectroscopy (XPS) [36]

XPS is an ex situ technique and requires high vacuum. XPS detects the energy of electrons that are emitted from inner-shell atomic orbitals after excitation with X-rays; the binding energies of the electrons have values that are distinct for each atom. The intensity of the spectra can be used to quantify the surface density of an alkanethiolate in a mixed SAM. It is difficult to extract molecular and kinetic information on the adsorbed species from such data. The use of X-rays in XPS may damage biological samples.

2.3.6. IR methods [110]

The analysis of data from attenuated total internal reflection FT-IR spectroscopy (ATR FT-IR) is model based and complex; it requires detailed knowledge of the IR spectrum and the conformation of the protein [111–114]. The spectra are modelled by taking into account spectroscopic selection rules at surfaces. Attenuation of amide band intensities are used to calculate the angle of β sheets or α helices with respect to the surface. The orientation of the whole protein on the surface may thus be inferred; inaccuracies in these determinations are common. Substrates for ATR FT-IR studies are typically materials whose surfaces are not modified easily (e.g. CdTe, Ge).

2.3.7. Low-angle X-ray reflectometry [115,116]

Low-angle X-ray reflectometry is sensitive to the variation in electron density at an interface and allows resolution of differences in packing among SAMs and protein layers [117]. The use of X-rays may at times cause radiation damage to the proteins.

2.4. Patterning of SAMs: soft lithography

Soft lithography is a set of techniques that allows convenient patterning and replicating (in polymers) of surfaces with features as small as 500 nm [7]. The key element of soft lithographic techniques is the use of alkanethiols, in conjunction with an elastomeric polymer with a pattern in bas-relief on its surface—a 'stamp' to transfer the pattern to the surface. The stamps are fabricated by casting and curing an elastomeric polymer, poly(dimethylsiloxane) (PDMS), against masters that consist of patterned photoresist supported on silicon substrates [118]. The masters are formed by photolithography using a conventional chrome mask for features between 500 nm and 50 μm. Features >20–50 μm can be generated using 'rapid prototyping'—a technique that uses a high resolution transparency produced by commercial printing as the mask for 1:1 contact projection photolithography [119].

Micro contact printing (μCP) is a technique that 'stamps' a pattern of SAM on the surface of the gold when an alkanethiol is used as 'ink' (Fig. 5A). [3,7,120] The stamps are wetted with a solution of alkanethiol, excess solvent is allowed to evaporate, and the stamp is placed on the gold substrates; because PDMS is an elastomer, it contacts the substrate conformally. As a result, a SAM forms in the pattern of the stamp. This process takes advantage of the ease with which alkanethiols react with a gold surface to form a relatively complete SAM (ca. 10 s for hexadecanethiol) [121]. Exposing the remaining bare gold substrate—that is the surface not covered by the printed SAM—to a second alkanethiol with a different terminal group allows patterning the surface into regions presenting two different terminal groups. The two-dimensional distribution of functional groups on the surface can control the ad-

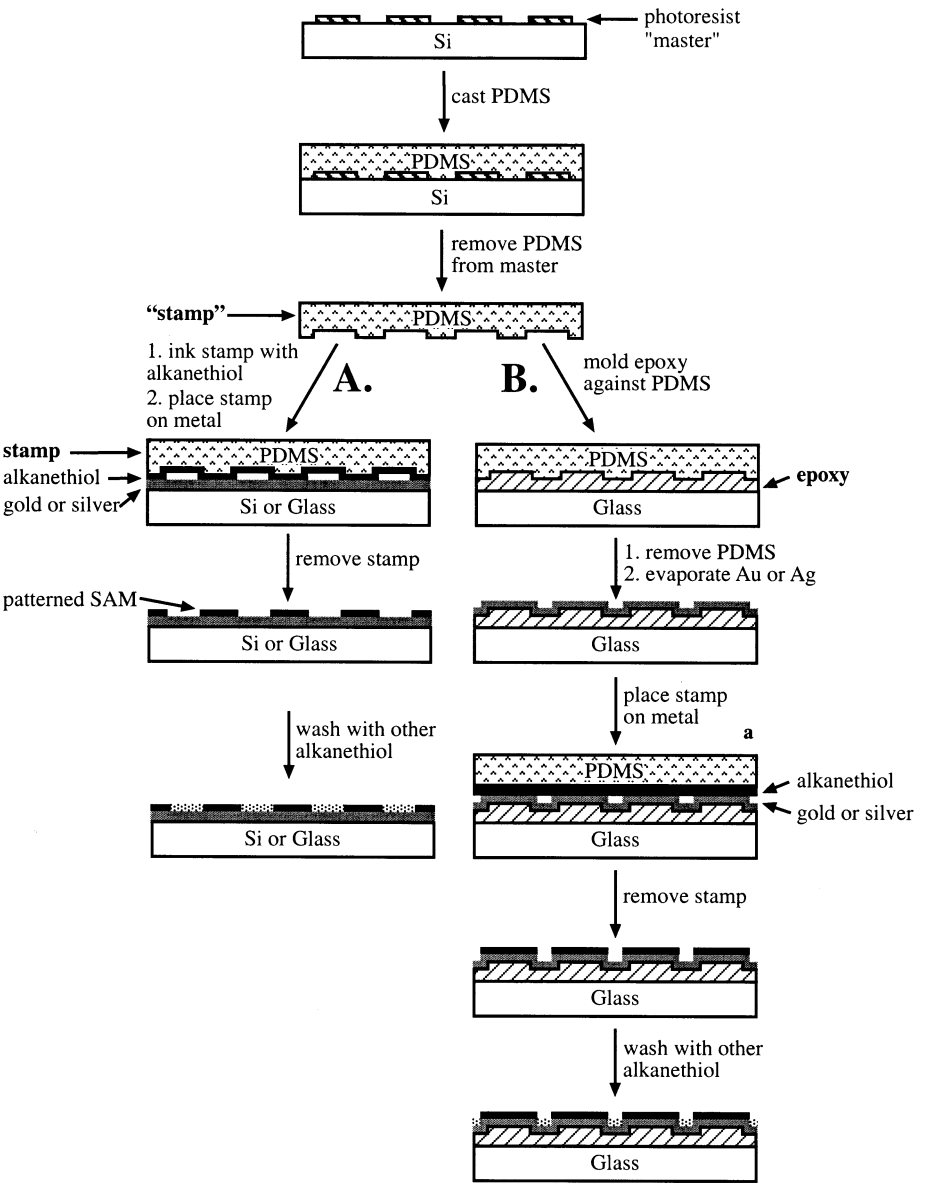


Fig. 5. Micro contact printing (μCP) and fabrication of contoured substrates using soft lithography. A silicon wafer coated with photoresist is exposed to UV light through a mask and developed to generate a relief pattern. Poly(dimethylsiloxane) (PDMS) is cast against this master to produce a stamp bearing a complementary relief structure. (A) The stamp is inked with an alkanethiol and placed on the gold surface; the pattern on the stamp is transferred to the gold by the formation of a SAM on the regions that contacted the substrate. The bare areas of the gold are exposed to a different alkanethiol to generate a surface patterned with a SAM that presents different chemical functionalities in different regions. (B) The PDMS stamp can also be used as a master to mold harder polymers and generate contoured surfaces. After evaporation of a layer of gold, these surfaces can be functionalized by μCP of one alkanethiol with a flat stamp. The grooves of the substrate can then be exposed to an alkanethiol presenting a different functional group to produce a contoured surface with patterned chemical reactivity.

sorption of proteins on the surface and, as a result, the attachment of cells to the substrates [11,12,14,61].

Chemically patterned surfaces can also be formed by μCP of amines onto surfaces that present interchain carboxylic anhydrides [65] or reactive ester groups [67] (Fig. 3A, B); a ligand R is then presented only in areas of the substrate that contacted the stamp. The remaining regions of the SAM can be patterned subsequently with a different alkylamine to generate a surface with spatially resolved chemical functionalities [64,65].

Surfaces can also be patterned with polymeric structures that are thicker (0.1–50 μm) than SAMs (2 nm). A stamp (called, in this context, a 'mold') with open channels is placed in contact with a surface; when a drop of prepolymer is placed at one of the edges of the stamps, capillary forces cause the prepolymer to move along and fill the channels defined by the mold [122–124]. The prepolymer is cured in situ, in contact with the PDMS mold; the mold is then removed to leave a polymeric structure on the surface (Fig. 5B) [123]. The success of this molding step rests on the ease with which the PDMS can be peeled away from the harder epoxy substrate; the ease of release of PDMS from micropatterned surfaces rests on a combination of its elastomeric properties and its low surface energy ($\gamma_{sv} + 21$ dyn/cm [125]). The pattern obtained in this way is the negative of the relief structure of the stamp.

Mrksich et al. used a variation of this method to devise a technique for fabricating contoured substrates and patterning alkanethiols onto them (Fig. 5B) for use in cell culture [13]. Epoxy prepolymer is molded against a PDMS master using reactive spreading and cured to generate a hard contoured surface; a transparent film of gold is then evaporated on the contoured epoxy substrate. The 'ridges' on the substrate are functionalized by μCP using flat stamps inked with an alkanethiol; the 'grooves' are then derivatized with a different alkanethiol. This procedure simultaneously controls the topography and the chemical properties of surfaces and is especially important in cell culture [13].

2.5. Non-covalent, physical interactions between proteins and SAMs

The first level of concern about the adsorption of proteins on synthetic surfaces focuses on non-biospecific adsorption: that is, adsorption reflecting hydrophobic or electrostatic interactions between the protein and the surface.

2.5.1. Hydrophobic surfaces

Previous studies of non-specific hydrophobic adsorption of proteins were performed on structurally heterogeneous polymeric surfaces whose hydrophobicity was varied by changing the extent of surface derivatization with a hydrophobic reagent [126–128]. SAMs make it possible to study this process rigorously by controlling the hydrophobicity of a surface at the molecular level. Mrksich et al. showed that the ellipsometric thickness and SPR response of layers of four different proteins adsorbed to SAMs increase as the hydrophobicity of the mixed SAM is increased (Fig. 6) [41]. This approach used mixed SAMs that comprise different mole fractions of hexa(ethylene gly-

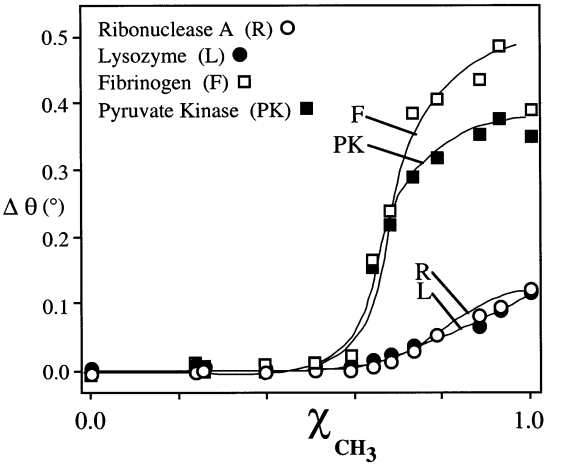


Fig. 6. Proteins adsorb to mixed SAMs made from undecanethiol and undecanethiol capped with $(EG)_6OH$ groups. The adsorption of the specified proteins was measured by SPR, in situ. The change in resonance angle ($\Delta\theta$) is plotted as a function of the mole fraction of undecanethiolate $\chi_{(CH_3)}$ in the SAM (measured by XPS) [41]. The thickness of the adsorbed layers of proteins increases in similar fashion as a function of $\chi_{(CH_3)}$ [41].

col) terminated undecylenic thiol and undecanethiol. The adsorption of protein was negligible until the mole fraction of methyl groups at the surface was greater than 0.5 (Fig. 6) [41].

We have extended the approach of Mrksich et al. to the study of the non-specific adsorption of proteins at hydrophobic interfaces. Hydrophobic interactions are studied best using a system in which the hydrophobic groups are structurally well-defined, and provide the only hydrophobic surfaces to which proteins adsorb. We measured the adsorption of proteins to SAMs that present different ratios of trityl-capped hexa(ethylene glycol) and tri- or hexa(ethylene glycol)-terminated alkanethiolate (Fig. 1) [129]. The amount of protein that adsorbed to the surface increased with the mole fraction of hydrophobic groups in the SAMs.

2.5.2. Charged surfaces

The interaction of proteins with charged surfaces is a subject of intense investigation [130–135]. The mechanism of electrostatic adsorption cannot be described simply by the interaction of two opposite charges: the details of the conformation and the chemical character of the protein have a strong effect on the adsorption process [136]. The charge densities of the surface and the protein, and the ionic strength of the aqueous buffer, largely determine whether adsorption will occur. Roth and Lenhoff found that the equilibrium constants for the adsorption of lysozyme and chymotrypsinogen A (both positively charged) to a negatively charged surface decrease with increasing ionic strength; the attraction between the proteins and the surface is screened and weakened by high values of the ionic strength [142]. Upon adsorption, the protein may undergo a conformational change that will also affect the strength of the interaction; unfolding of the tertiary structure may lead to a change in the density of charged groups and their interaction with the surface [137].

In the past, the interpretation of results from experiments performed on charged surfaces has also been complicated by the use of substrates that present hydrophobic groups that interact favorably and non-specifically with the protein. It is difficult to deconvolute the contributions of these two effects to the adsorption [138].

Mixed SAMs on gold make it possible to control the distribution of charges on a surface that otherwise resists the adsorption of protein. Hallock and co-workers monitored the adsorption of a charged polymer to mixed SAMs of $^{-}S(CH_2)_{10}CH_3$ and $^{-}S(CH_2)_{10}COOH$ by SPR; they found that the amount of polymer that adsorbs to the SAMs undergoes an abrupt increase upon increasing the mole fraction of alkanethiolate that presents carboxylic acid groups [139]. Hallock et al. propose that the increasing mole fraction of carboxylic acid groups causes the distance between negative charges on the surface to match the distance between positive charges on the polymer—a form of templating [139]. To this date, studies of the interaction of biological macromolecules with charged surfaces formed with SAMs have involved only single types of alkanethiols. Corn and co-workers used SPR to monitor the electrostatic adsorption of streptavidin and poly-L-lysine onto carboxylic acid terminated SAMs; these surfaces were functionalized and used to immobilize biomolecules rather than to study electrostatic interactions [97,140–143].

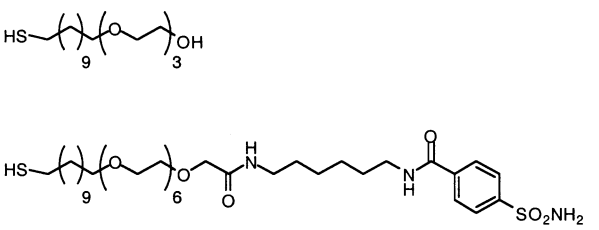
2.6. Non-covalent, biospecific interactions between biomolecules and SAMS

2.6.1. Carbonic anhydrase and derivatives of benzenesulfonamide

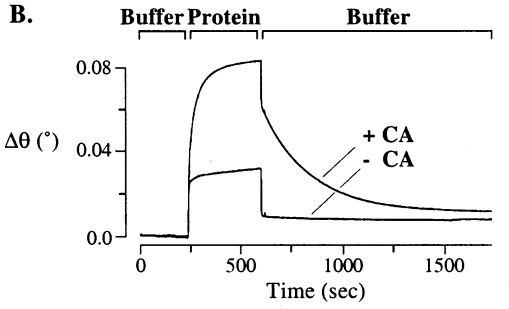
This system of enzyme and inhibitors is a model that is representative of simple, monovalent protein–ligand interactions, and that is convenient to use in studying the interactions of biomolecules with surfaces. Mrksich et al. have described studies of the interaction of bovine carbonic anhydrase (BCA) biospecifically with mixed SAMs presenting benzenesulfonamide (a specific ligand for the protein) and EG_3OH groups (Figs. 1 and 7A) [23].

Association of BCA with benzenesulfonamide ligands in these SAMs is $>90\%$ reversible (Fig. 7B, C) [23]. The interfaces generated using the benzenesulfonamide ligand show high specificity for BCA. The SPR response measured when this SAM was placed in contact with a solution con-

A.



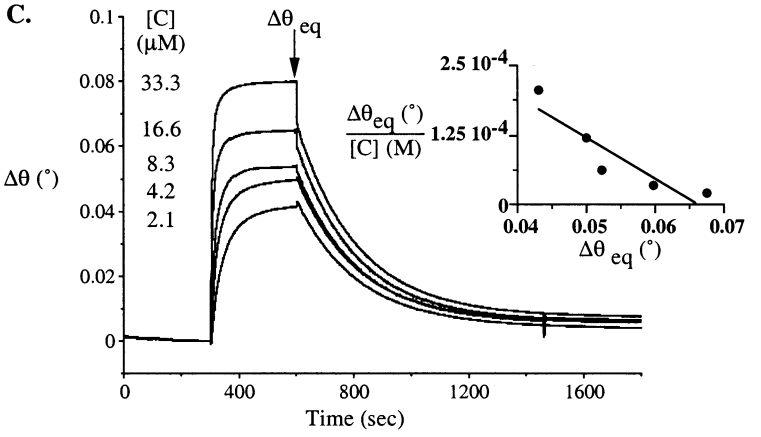
B.



Nonspecific Proteins

Fibrinogen
Myoglobin
Alcohol Dehydrogenase
Trypsin Inhibitor
Acylase I
α-Lactalbumin
Cytochrome C
β-Casein
RNase A

C.



D.

	K_d (μM) (Scatchard Plot)	k_{on} (M^{-1} s^{-1})	k_{off} (s^{-1})	K_d (μM) (calc. k_{off} / k_{on})
Solution 1	**0.05**	–	–	–
Solution 2	**0.63**	**112000**	**0.07**	**0.63**
Dextran Matrix	**1.5**	**7600**	**0.0052**	**0.68**
Mixed SAM	–	**19000**	**0.0054**	**0.28**
Common Intermediate	**1.4**	**16400**	**0.0048**	**0.29**

Fig. 7. (*Continued*)

taining several proteins that do not bind benzenesulfonamide biospecifically increased 3-fold in the presence of BCA (Fig. 7B). The amount of protein that adsorbed to the SAM non-specifically was negligible (Fig. 7B, C) [23].

Mrksich et al. found that the length and chemical properties of the moiety that linked the benzenesulfonamide ligand to the $(EG)_6$ layer were important in designing the biospecific SAM. Benzenesulfonamide should be bound to the $(EG)_nOH$ layer through a linker whose length allows it to reach the deep binding site of the protein. Since the active site of BCA is ≈ 15 Å from the surface of the protein [144], Mrksich et al. used a hexyl moiety as a linker.

The values of the equilibrium dissociation constant of BCA with benzenesulfonamide on SAMs and with benzenesulfonamide derivatives in solution are comparable; $K_d = 2.8 \times 10^{-7}$ ($k_{on} = 1.9 \times 10^4$ $M^{-1}s^{-1}$, $k_{off} = 5.4 \times 10^{-3}$ s^{-1}) for a SAM presenting benzenesulfonamide, while in solution $K_d = 5 \times 10^{-8}$ [23]. The relatively small difference (a factor of six in this comparison) between these values of K_d may arise from unfavorable steric interactions at the surface of the SAM [23,145].

2.6.2. Vancomycin and derivatives of D-Ala-D-Ala

We have used anhydride surfaces to immobilize D-Ala-D-Ala (DADA) on a SAM in order to study the interaction of vancomycin with this fragment of the bacterial cell wall [146,147]. Rao et al. found that although monomeric vancomycin binds to a SAM with a value of the equilibrium dissociation constant (Fig. 8A, $K_D = 1.1$ μM) similar to that obtained in solution ($K_D = 2$ μM), the individual values of k_{on} and k_{off} on the SAM differ from those of solution by a factor of 400. Fig. 7B shows that the rate constants for the interaction of a vancomycin dimer with DADA groups on the surface are affected by mass transport. As the concentration of vancomycin dimer decreases, the SPR signal becomes linearly dependent on time; this behavior is typical of mass transport-limited processes (Fig. 8B).

2.7. Covalent immobilization of proteins on SAMs

It is sometimes necessary to attach proteins covalently to a synthetic surface: applications include sensors, biocatalytic electrodes, and catalysts. The formation of bioactive surfaces requires that the biological molecule of interest be immobilized in a manner that preserves its activity and specificity. Covalent attachment of a biological molecule to a surface should, ideally, result in a known orientation of the active site that does not obstruct its interaction with ligands: no method presently accomplishes this objective with every protein. We describe several techniques that achieve this goal with small groups of proteins, using complexation with surface-bound metal atoms or reaction with patterned reactive groups.

Fig. 7. Specific interactions between biomolecules and ligands presented at the surface of SAMs. (A) Structures of the alkanethiols used to form SAMs for the studies summarized in part (B). (B) SPR sensorgrams illustrating the binding of BCA to a mixed SAM presenting as ligands (L) benzenesulfonamide groups with $\chi_L \sim 0.03$) and $(EG)_3OH$ groups. This mixed SAM does not allow the adsorption (lower curve) of proteins other than BCA (each at 0.2 mg/ml). In the presence of BCA (5 μM), SPR readily measures the adsorption of protein (upper curve). (C) Sensorgrams obtained by flowing BCA (at the indicated concentrations) over a SAM with $\chi_L \approx 0.01$: the ligand was immobilized using the common intermediate approach described in Fig. 3B. The inset shows a Scatchard plot that was used to find the value of K_d: the change in the equilibrium value of the signal ($\Delta\theta$eq) on adsorbing protein is divided by the concentration of protein ([C]) and plotted as a function of $\Delta\theta$eq. D) The values of kinetic constants measured using a variety of immobilization chemistries are tabulated. Lahiri et al. [66] compared the binding of BCA to benzenesulfonamide that had been immobilized using NHS/EDC chemistry on a dextran matrix (commercially available from Biacore), and on a mixed SAM formed with the method described in Fig. 3B [145]. Mrksich et al. measured the kinetic parameters for binding of BCA to a mixed SAM comprising benzenesulfonamide groups and $(EG)_3OH$ groups [23]. The 'solution 1' value of K_d was obtained from a Scatchard analysis of CA binding to soluble $CH_3O(EG)_6CH_2CONH(CH_2)_6NHCO(C_6H_4)SO_2NH_2$. The 'solution 2' values were obtained by Taylor et al. with soluble $CH_3NHCO(C_6H_4)SO_2NH_2$ and human carbonic anhydrase: the rate constants of association and dissociation were obtained with a stopped-flow apparatus, the equilibrium constant with a fluorescence titration [196].

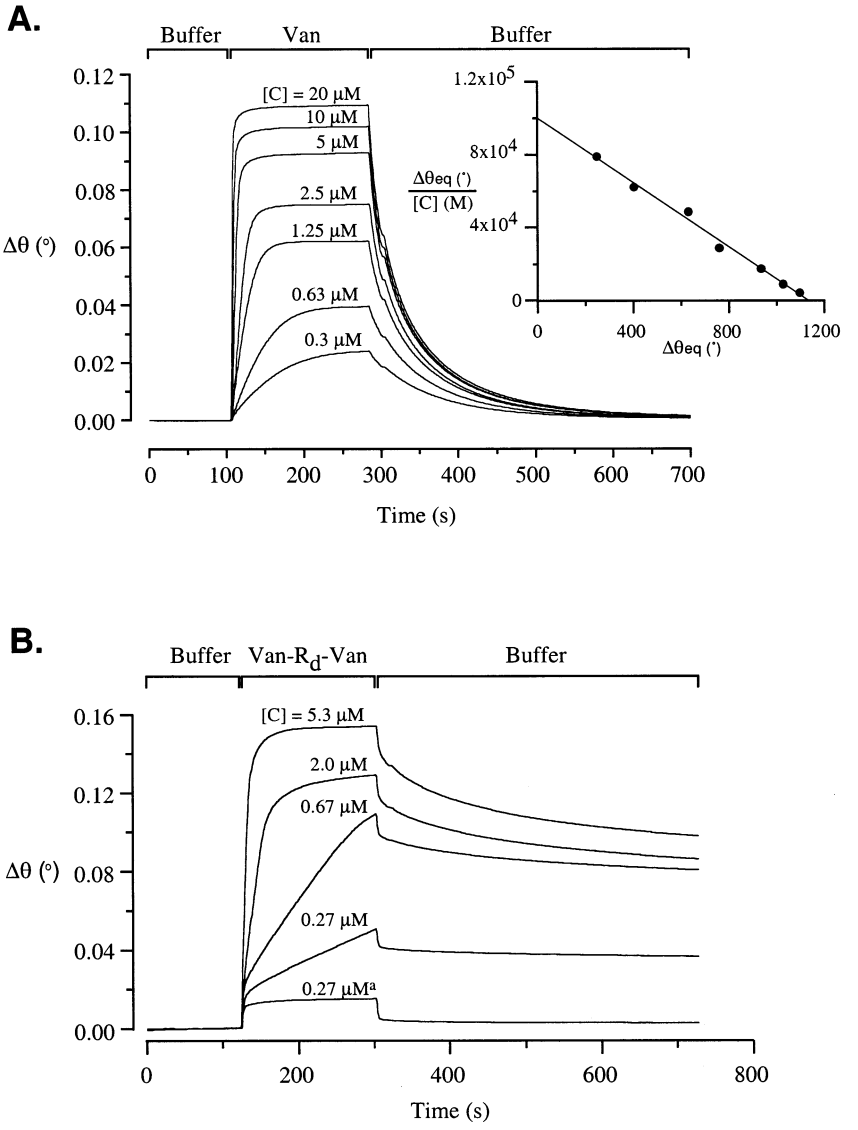


Fig. 8. (A) The binding of different concentrations of vancomycin ([C] as indicated on the plot) to a mixed SAM presenting an equimolar mixture of D-Ala-D-Ala and carboxylic acid groups that was formed according to the method described in Fig. 3A [146]. The inset is a Scatchard plot of the data; $\Delta\theta_{eq}$ is the value of the response after the system reaches equilibrium. (B) The binding of different concentrations of a vancomycin dimer (Van-R_d-Van) ([C]) to a mixed SAM is indistinguishable from that used in (A). As the concentration of the analyte decreases, the rate of mass transport to the surface also decreases and the sensorgrams take on a shape that is indicative of mass transport-limited kinetics (see text). [a]In the control experiment, the SAM presented only carboxylic acid groups.

2.7.1. Immobilization of coordination complexes: His-tagged proteins and derivatives of nitrilotriacetic acid (NTA)

Recombinant proteins are often tagged with a stretch of six histidines (His-tag) to facilitate purification using metal-affinity chromatography. The imidazole nitrogen atoms of the histidine groups bind metal atoms in a suitable co-ordination environment; these co-ordination sites can be placed on a surface. Nickel(II) (as a nitrilotri-

acetic acid complex) co-ordinates well with histidines; this association allows the immobilization of biomolecules that bear a His-Tag sequence. Sigal et al. developed a SAM terminated with a nitrilotriacetic acid (NTA-SAM) chelating agent that binds Ni(II) and the His-Tag motif selectively and tightly (Fig. 1). SPR studies confirm that SAMs presenting NTA ligands enabled the selective immobilization of His-tagged proteins (human TATA box binding protein, transcriptional activator Gal 4, two components of the yeast RNA polymerase II holoenzyme, and a single-chain T-cell receptor construct) [40] through complexation of the Ni(II) atom by two histidines. Sigal et al. obtained stable monolayers of immobilized His-tagged proteins that were recognized by antibodies. The His-tagged proteins could be removed from the surface using solutions of imidazole; the same surface could then be used to immobilize a different protein for subsequent studies [40].

2.7.2. Examples of immobilization strategies from the literature

Many biomolecules have been immobilized on gold by their functionalization with thiol groups [148,149]. This approach has been particularly useful for the development of biosensors that rely on the modification of electrodes with enzymes [148]. Direct attachment of a biomolecule to a surface using its reactivity for the substrate does not offer the ability to control the surface density and the environment around the molecule of interest. The molecular-level control over surface properties that is possible with SAMs allows the development of efficient methods for the presentation of ligands at the surface. The ideal method for the immobilization of a protein should involve the attachment of the protein at a surface that is otherwise inert to the adsorption of biomolecules. The techniques we have described (Fig. 3B) achieve this goal with proteins that have amino groups accessible on their surface [117].

Patel et al. used mixed SAMs formed from 3-mercaptopropanoic acid and 11-mercaptopropanoic acid to immobilize catalase using NHS/EDC chemistry; the resulting orientation of the protein could not be specified a priori [25]. The charged character of the surface did not prevent entirely the non-specific adsorption of the protein [25]. Biebuyck et al. have used spreading guided by the channels in a PDMS stamp to pattern the covalent attachment of antibodies to SAMs for the development of immunosensors [42,43]. They placed PDMS stamps on gold substrates functionalized with SAMs presenting NHS active esters. An antibody, in solution, was introduced into the channels, where it reacted covalently with the surface-bound active ester. Corn and co-workers adsorbed poly-L-lysine electrostatically onto carboxylic acid terminated SAMs and functionalized the polymer with a heterobifunctional linker that allowed the covalent immobilization of DNA probes; SPR imaging was used to detect hybridization of DNA at the surface [141,143]. Herne et al. formed mixed monolayers on gold with mercaptohexanol (MCH) and a DNA strand modified with a thiol group; MCH removes DNA strands that adsorb to the gold non-specifically and improves the hybridization efficiency [149]. Peterlinz et al. used a home-built two-wavelength SPR instrument to study the kinetics of hybridization of DNA in SAMs made with Herne's technique [26,98]. The hybridization data fit a diffusion-limited Langmuir adsorption model, and the melting temperature increased in the absence of NaCl [26].

2.8. Interactions of cells with SAMs

Most mammalian cells live in contact with other cells and the surfaces of tissues [150] These contacts contribute to the set of stimuli required by the cell for stable, long-term metabolism. Many mammalian cell types will quickly undergo apoptosis (programmed suicide) if they are not in contact with a surface that promotes their adhesion [150–153]. Cells must also adhere and spread onto a substrate in order to proliferate. When cells proliferate to a point where they crowd each other on a surface, their growth stops. It is presently unclear how cells sense these features of their environment, and how they transduce that information into the signals that regulate metabolism and proliferation. It would therefore be useful to have a system that could be used to

study the interactions of cells with surfaces in vitro. This system might help both to understand the mechanisms that the cell uses to sense its environment and to assist in the design of materials and devices. This type of application is representative of those that require the ability to present complex ligands at a surface in patterns and to thereby direct the adhesion of cells to the surface.

There are a number of other applications that involve patterning the adhesion and growth of cells on surfaces. In one, living cells are used as sensors for toxins [154–157]. These types of cell-based assays have been known for several years, but a number of improvements in the technology are required before they are truly practical; packaging, long-term storage, and signal readout of cell-based materials are the most important, unsolved, issues. High throughput screening and the analysis of combinatorial libraries sometimes rely on cell-based assays; such analyses might be faster and require smaller quantities of materials if there were a practical way of patterning cells into an integrated micro-analytical device that would isolate cells from one another and enable each cell to be treated with a separate analyte [158–161]. Such systems would enable the rapid and parallel screening of libraries of compounds in an assay that could be analyzed using conventional microscopic detection.

Many applications of patterned cells have involved unmodified silicon [160–163] and SAMs of alkylsiloxanes on glass or silicon [154–157,164–169]. The range of surface chemistries available using these techniques is narrow. Trichloroalkylsilanes chemisorb on the surface of SiO_2 to form SAMs of alkylsiloxanes. Alkylsiloxane SAMs are less easy to form than those of alkanethiolates on gold, and a variety of organic functional groups are not compatible with the conditions used for their formation. SAMs of siloxanes can be patterned using deep UV photolithography. Photolithographical pattern transfer works well in certain cases, but it provides only limited control over the chemical and physical properties of the surface; it is also technically complicated, and difficult to use over large areas and curved surfaces [170]. Although SAMs of alkanethiolates are better-suited for model studies of the interaction of cells with surfaces, SAMs of alkylsiloxanes are more widely applicable to existing technological processes; such SAMs can be formed on a variety of surfaces that include metals, polymers, and silica. Table 3 lists several types of cells that have been patterned with SAMs of alkylsiloxanes.

2.8.1. Control of surface chemistry

Hydrophobic SAMs patterned on flat gold substrates by μCP allow the patterned non-specific adsorption of extracellular matrix (ECM) proteins (fibronectin, fibrinogen, vitronectin, collagen I and IV, and laminin) that promote the adhesion of different cell types (bovine capillary endothelial, rat hepatocytes, mammalian carcinoma, and wild type and transfected chinese hamster ovary cells) (Fig. 9) [10–14,61,171,172]. The use of SAMs that present tri(ethyleneglycol) groups at the surfaces of the substrates (Fig. 4) ensures that if the cells secrete their own ECM proteins, they will not adsorb outside of the printed features at least for seven days (Fig. 9) [11,13,44,173,174]. Proteins can also be printed directly onto substrates by pcp [175,176]

An alternative approach to the control of the chemistry of a surface for studies of cell adhesion involves the use of mixed SAMs of alkanethiolates that present EG_3OH groups and biologically active groups. A ligand (a short peptide (R), for example) can be attached to an alkanethiol—$HS(CH_2)_{11}EG_6OR$; this alkanethiol can be used to form mixed SAMs with an alkanethiol that presents EG_3OH groups [19]. These systems ensure

Table 3
Types of cells patterned with SAMs of alkylsiloxanes

Cell type	Ref.
Hippocampal neurons (rat)	[155,157,164,167,168]
Neuroblastoma (human, SK-N-SH)	[156]
Hepatocytes (rat)	[166]
Fibroblasts (rat, 3T3)	[166]
Aortic endothelial (porcine)	[164]
Umbilical vein endothelial (human)	[165]
Cerebral microvascular endothelial (porcine)	[165]

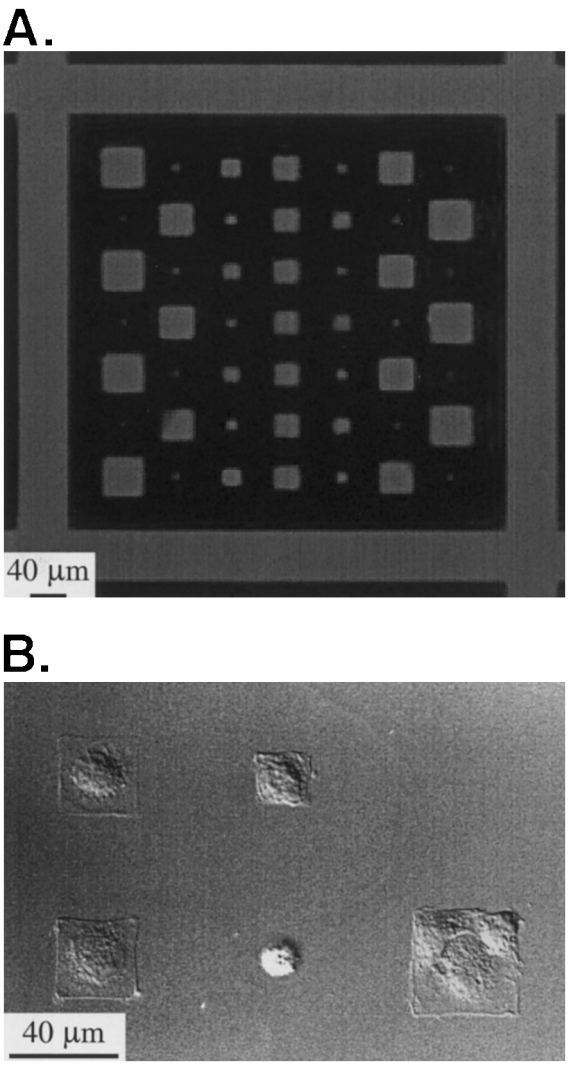


Fig. 9. (A) A gold surface was patterned into regions of hexadecanethiolate and undecanethiolate terminated with tri(ethylene glycol) by μCP. Fibronectin (light) adsorbed on the hydrophobic squares of hexadecanethiolate but not on the tri(ethyleneglycol) terminated alkanethiolate (dark). Patterned substrates were soaked in a solution of fibronectin (50 μg/ml in phosphate buffered saline (PBS)) for 2 h, fixed using 20% paraformaldehyde (v/v) in PBS buffer and then immersed in a solution of anti-human fibronectin IgG (5 μg/ml) for 1 h followed by extensive rinsing. The substrates were then placed in contact with 100 μl of Texas Red®-labeled goat anti-rabbit IgG (50 μg/ml) for 1 h, followed by mounting in fluoromount-G (Southern Biotechnology Inc.). (B) Bovine capillary endothelial (BCE) cells patterned by culturing on a substrate presenting hydrophobic squares of varying sizes that were coated with fibronectin, prior to incubation with cells, using the procedure described in (A).

that the adhesion of a cell to the SAM will be caused *only* by its interaction with this ligand; the presence of EG_3OH groups prevents the adsorption of ECM proteins that the cell may secrete as it attempts to remodel the surface.

2.8.2. The influence of contact between cell and substrate on cell metabolism

Using μCP to pattern regions of hydrophobic SAMs, it has been possible to study the influence of size, shape, and separation of adhesive islands of ECM proteins on the growth of bovine capillary endothelial (BCE) cells (Fig. 9). We have generated attached BCE cells that are round and square (Figs. 9 and 10) by allowing them to adhere to hydrophobic islands (generated by μCP as described in Figs. 5 and 9) that were coated with ECM proteins and shaped in the form of circles and squares [11]. We also patterned cells across multiple islands (3–5 μm diameter) (Fig. 10).

Variation of the surface area to which a cell is confined influences the onset of different genetic programs. When monitoring the onset of growth and apoptosis, Chen et. al. found that cell growth is turned on by the area covered by the spread cell rather than by the area of the adhesive surface [11]. The apoptotic index of cells (defined as the number of cells entering apoptosis divided by the total number of adherent cells) decreased as the size of the square island on which they were cultured increased from 5 to 50 μm, while DNA synthesis—a measure of growth—increased [11]. These results are consistent with two hypotheses: (i) that growth increases with the area of the surface that is in adhesive contact with the cell; and (ii) that growth depends on the extent of cell spreading, rather than the area of the adhesive contact. Chen et al. tested this hypothesis by culturing cells on substrates that presented islands with diameters of 20, 5, and 3 μm separated by 40, 10 and 6 μm respectively (Fig. 10). Single cells adhered to these substrates such that the total area of ECM contacted by each cell was constant. DNA synthesis did not scale with the contact area but with the *projected* cell area. Growth was almost completely inhibited in cells cultured on 20 μm circles (where cells were least spread), while it

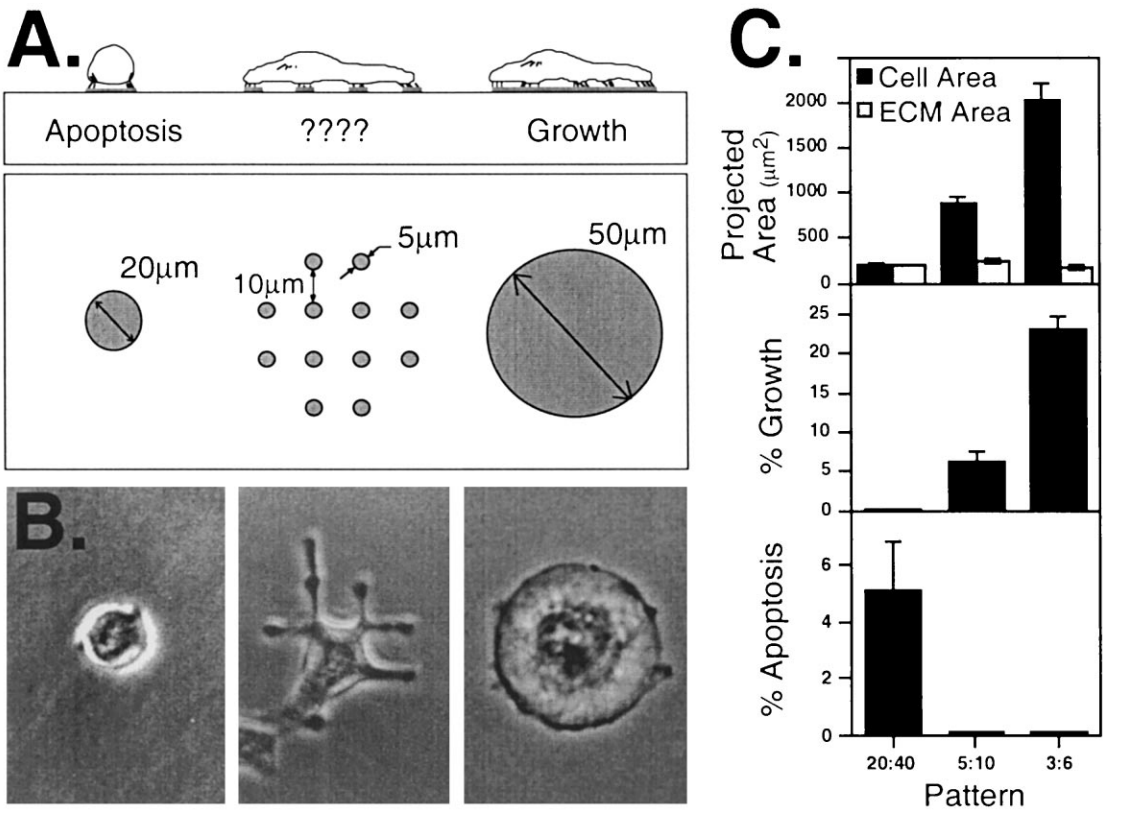


Fig. 10. (A) Schematic illustration of substrates used to vary the projected area and the contact area of BCE cells growing on patterned SAMs of alkanethiolates on gold. (B) Optical micrographs of cells spread on the patterns shown in (A) fabricated according to the procedures described in Fig. 9. (C) On such substrates, cells contact an area of the ECM that is predefined by the pattern, but they are capable of adhering to neighboring islands of ECM and spreading to an area greater than that of the sum of their contacts. The graph plots values of the projected cell area and ECM contact area, growth index, and the apoptotic index (percentage of cells that enter apoptosis) for cells cultured on circular islands (pattern) having diameters of 20, 5, and 3 μm that were separated by 40, 10, and 6 μm, respectively [11].

was largest for cells on 3 μm circles spaced by 6 μm (where spreading was largest) [11]. Cells patterned on 5 μm circles (10 μm spacing) adhered to the surface and had intermediate values of the growth and apoptotic indices. These results indicate that in spreading, the *aggregate* area covered by a cell—including the areas to which it adheres and those to which it is not attached—is the major determinant of a cell's proliferation.

2.8.3. Biospecific interactions: SAMs presentzag the GRGD groups

Adsorption of ECM proteins to hydrophobic regions provides a surface that promotes adhesion of cells. It is, however, difficult to control the conformation and orientation of the adsorbed ECM protein, and therefore the density (and perhaps the conformation) of the ligands. Integrin receptors on the surface of BCE cells bind the arginine-glycine-aspartate (RGO) amino acid sequence found in fibronectin (FN) and other ECM proteins. [177,178]. A number of studies have shown that surfaces that present *only* RGD groups direct the adhesion and spreading of mammalian cells [179–181]. These studies were performed on surfaces that did *not* resist the adsorption of adhesive proteins secreted by the cells; [18] with remodeling, over time, the molecu-

lar composition of the surface and the type of membrane-bound receptors that bind to the surface change in ways that cannot be controlled. These systems did not allow the rigorous study of the effects of cell–ligand interactions on biological processes.

Roberts et al. developed a system of SAMs of alkanethiolates that present EG_6OGRGD groups, to promote the adhesion of cells, and EG_3OH groups, to resist the adsorption of proteins secreted by the cell. These surfaces supported the adhesion and the survival of cells, and prevented the cells from remodelling the surface, during a period of ≈ 24 h. Mole fractions of EG_6OGRGD in the SAM as low as 0.00001 promoted the adhesion of cells; maximum spreading of cells was observed with mole fractions ≥ 0.001. Cells cultured for 4 or 24 h on mixed SAMs presenting EG_6OGRGD could be released from the surface by brief exposure to a soluble ligand that contained the RGD peptide (Fig. 11) [19]. This reversal of cellular adhesion had been demonstrated previously by Sims et al. [182] for RGD-coated glass, but it was effective only for cells that had been attached to the substrate for short times (2–4 h). Cells that adhered to SAMs presenting GRGD groups apparently did not deposit a functional matrix on the protein-resistant EG_3OH background. These cells could be released from the surface by the addition of a soluble, RGD-containing, ligand that competed with the RGD present on the surface [19]. Cells adhered more tightly to a layer of fibronectin adsorbed on an hydrophobic SAM (measured by the time necessary to release the cells from the surface with a soluble RGD-containing ligand) than to a SAM presenting GRGD because they were able to remodel that surface; this problem was circumvented by the use of mixed SAMs that also presented EG_3OH groups that resisted the adsorption of protein. A mixed SAM that presented GRGD ligands (with $\chi < 0.1$) was sufficient to support the adhesion of cells and prevent the cells from remodeling the surface. These mixed SAMs may find applications in experiments that require the release of cells from surfaces using mild reagents that do not damage the cell surface (for example, the commonly used proteases) (Fig. 11).

2.8.4. The adhesion of cells to contoured substrates [13]

Several groups have demonstrated that the topography of a substrate can be an important determinant of cellular behavior [183–186]. These results were obtained with silicon substrates that were fabricated using photolithographic techiques that are not available to many biologists. Contoured substrates affected the alignment of mammalian cells, [184,186] while fibroblasts cultured on grooves with V-shapes synthesized and secreted more fibronectin than cells cultured on

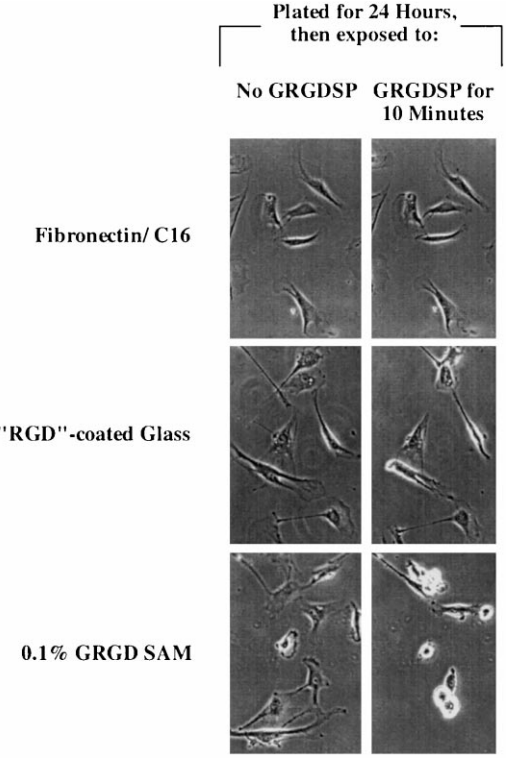


Fig. 11. Attached BCE cells retract and release from the surface when they are exposed to soluble GRGD. We show micrographs of cells that were cultured for 24 h on the indicated substrates before (left column) and after (right column) being exposed to soluble GRGDSP ligand for 10 min. Fibronectin/C16 refers to the surface of a hydrophobic SAM ($S(CH_2)_{15}CH_3$, C16) on gold that was covered by fibronectin; 'RGD'-coated glass refers to a cover slip that was immersed in a solution of a 20 amino acid fragment that contained RGD. Only cells that were cultured on a SAM on gold presenting 0.1% GRGD ligand at a surface that otherwise presented $(EG)_3OH$ groups retracted to a detectable extent (see text).

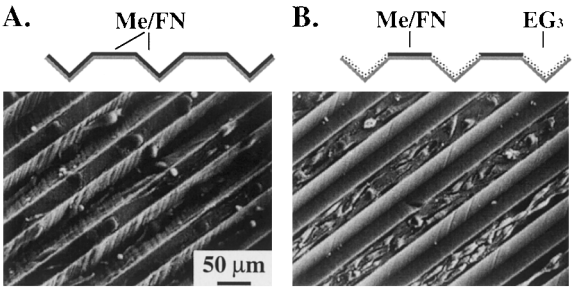


Fig. 12. Scanning electron micrographs of endothelial cells patterned on ridges and grooves 25 μm wide. These substrates were produced using a slight modification of the scheme outlined in Fig. 4B to form grooves that were triangular in shape that were then coated with a layer of titanium (to promote the adhesion of gold) and a layer of gold [41]. The ridges of this substrates were functionalized by μCP hexadecanethiol with a flat stamp; the substrates were then exposed to a solution of an alkanethiol to functionalize the grooves of the substrates. Fibronectin was adsorbed onto these substrates following the procedure described in Fig. 8A. (A) Grooves were functionalized with hexadecanethiolate; fibronectin, and consequently cells, were deposited onto the entire contoured substrate. (B) Only the plateaux presented a hydrophobic surface for the adsorption of fibronectin; the grooves were functionalized with a tri(ethylene glycol)-terminated alkanethiol that resisted the adsorption of fibronectin and the adhesion of cells [13].

flat substrates [183]. The techniques we have described in Fig. 5B have been used to adsorb proteins and grow cells onto contoured polymeric substrates (Fig. 12) that were patterned with SAMs.

3. Conclusions

SAMs provide excellent model systems for the study of the interactions of proteins and cells with surfaces. SAMs provide interfaces whose physical and chemical properties can be tailored easily at the molecular level by incorporating the desired functionalities into the terminal position of the alkanethiols and forming mixed SAMs. They are sufficiently stable for use in model studies of the adsorption of proteins (biospecific and non-specific) and of the adhesion of cells to surfaces. Non-adsorbing surfaces are essential to the rational development of biosurfaces. The formation of mixed SAMs with alkanethiols that present $(EG)_3OH$ groups and $(EG)_6OR$ groups (where R is the ligand of interest) makes it possible to study adsorption or binding events of complex biological media *only* with the ligand of interest without the interfering effects of the layer that supports the ligand. The kinetics and the thermodynamics of the interactions of biomolecules (especially proteins) with surfaces can be studied in situ using SPR. We and others have used this technique to study successfully the biospecific and the non-biospecific adsorption of proteins at surfaces. These basic studies provide clues about the parameters that influence adsorption and may aid in the design of materials with improved performance.

The ability to pattern the adsorption of proteins by patterning SAMs on gold provides a valuable tool in studying the attachment of cells to surfaces. The effects of the chemistry and the topology of a surface on cellular metabolism have been studied with such systems. The combination of SAMs, inert surfaces, μCP, and SPR, forms a powerful set of tools for modifying surfaces and for characterizing the interfacial processes in which they engage. The model systems described herein will further our understanding of biointerfaces and our ability to test hypotheses and design materials.

The combination of these separate sets of materials and techniques—SAMs, soft lithography, inert surfaces, and SPR—is strongly synergistic. Collectively, they enable molecular events occurring at the interface between biological media and synthetic surfaces to be studied in useful detail, and they point the way to techniques that will open the way to new methods of interrogating the cell about its interactions with its environment.

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References

[1] G.M. Whitesides, G.S. Ferguson, Chemtracts 1 (1988) 171–187.
[2] G.M. Whitesides, C.B. Gorman, in: A.T. Hubbard (Ed.), Handbook of Surface Imaging and Visualization, CRC Press, Boca Raton, 1995, pp. 713–733.
[3] M. Mrksich, G.M. Whitesides, Trends Biotechnol. 13 (1995) 228–235.
[4] M. Mrksich, G.M. Whitesides, Ann. Rev. Biophys. Biomol. Struct. 25 (1996) 55–78.
[5] A. Kumar, H.A. Biebuyck, G.M. Whitesides, Langmuir 10 (1994) 1498–1511.
[6] A. Kumar, N.A. Abbott, E. Kim, H.A. Biebuyck, G.M. Whitesides, Acc. Chem. Res. 28 (1995) 219–226.
[7] Y. Xia, G.M. Whitesides, Annu. Rev. Mater. Sci. 28 (1998) 153–184.
[8] K.L. Prime, G.M. Whitesides, J. Am. Chem. Soc. 115 (1993) 10714–10721.
[9] G.P. López, H.A. Biebuyck, G.M. Whitesides, Langmuir 9 (1993) 1513–1516.
[10] G.P. López, H.A. Biebuyck, R. Härter, A. Kumar, G.M. Whitesides, J. Am. Chem. Soc. 115 (1993) 10774–10781.
[11] C.S. Chen, M. Mrksich, S. Huang, G.M. Whitesides, D.E. Ingber, Science 276 (1997) 1425–1428.
[12] G.P. López, M.W. Albers, S.L. Schreiber, R.W. Carroll, E. Peralta, G.M. Whitesides, J. Am. Chem. Soc. 115 (1993) 5877–5878.
[13] M. Mrksich, C.S. Chen, Y. Xia, L.E. Dike, D.E. Ingber, G.M. Whitesides, Proc. Natl. Acad. USA 93 (1996) 10775–10778.
[14] R. Singhvi, A. Kumar, G.P. Lopez, G.N. Stephanopolous, D.I.C. Wang, G.M. Whitesides, D.E. Ingber, Science 264 (1994) 696–698.
[15] J.D. Andrade, V. Hlady, Adv. Polym. Sci. 79 (1986) 1–63.
[16] T.A. Horbett, J.L. Brash, in: J.L. Brash, T.A. Horbett (Eds.), Proteins at Interfaces: Physicochemical and Biochemical Studies, American Chemical Society, Washington, DC, 1987, pp. 1–36.
[17] J.J. Ramsden, Q. Rev. Biophys. 27 (1993) 41–105.
[18] S. Hakomori, M. Fukuda, K. Sekiguchi, W.G. Carter, in: K.A. Piez, A.H. Reddi (Eds.), Extracellular Matrix Biochemistry, Elsevier, New York, 1984.
[19] C. Roberts, C.S. Chen, M. Mrksich, V. Martichonok, D.E. Ingber, G.M. Whitesides, J. Am. Chem. Soc. 120 (1998) 6548–6555.
[20] L.H. Dubois, R.G. Nuzzo, Annu. Rev. Phys. Chem. 43 (1992) 437–463.
[21] A. Ulman, Chem. Rev. 96 (1996) 1533–1554.
[22] A.R. Bishop, R.G. Nuzzo, Curr. Opin. Colloid Interface Sci. 1 (1996) 127–136.
[23] M. Mrksich, J.R. Grunwell, G.M. Whitesides, J. Am. Chem. Soc. 117 (1995) 12009–12010.
[24] L. Haussling, H. Ringsdorf, F.-J. Schmitt, W. Knoll, Langmuir 7 (1991) 1837–1840.
[25] N. Patel, M.C. Davies, M. Harshorne, R.J. Heaton, C.J. Roberts, S.J.B. Tendler, P.M. Williams, Langmuir 13 (1997) 6485–6490.
[26] K.A. Peterlinz, R.M. Georgiadis, T.M. Herne, M.J. Tarlov, J. Am. Chem. Soc. 119 (1997) 3401–3402.
[27] J. Spinke, M. Liley, F.-J. Schmitt, H.-J. Guder, L. Angermaier, W. Knoll, J. Chem. Phys. 99 (1993) 7012–7019.
[28] C. Pale-Grosdemange, E.S. Simon, K.L. Prime, G.M. Whitesides, J. Am. Chem. Soc. 113 (1991) 12–20.
[29] K.L. Prime, G.M. Whitesides, Science 252 (1991) 1164–1167.
[30] K.L. Prime, Y.-H. Chu, W. Schmid, C.T. Seto, J.K. Chen, A. Spaltenstein, J.A. Zerkowski, G.M. Whitesides, Macromolecular assemblies, in: P. Stroeve, A.C. Balazs (Eds.), ACS Symposium Series, American Chemical Society, Washington, DC, 1992, pp. 227–239
[31] H. Raether, in: G. Hass, M. Francombe, R. Hoffman (Eds.), Physics of Thin Films, Academic Press, New York, 1977, pp. 145–261.
[32] R. Karlsson, H. Roos, L. Fagerstam, B. Persson, Methods: A Companion to Methods in Enzymology, 1994, 6, pp. 99–110.
[33] M. Fivash, E.M. Towler, R.J. Fisher, Curr. Opin. Biotechnol. 9 (1998) 97–101.
[34] P. Schuck, Ann. Rev. Biophys. Biomol. Struct. 26 (1997) 541–566.
[35] A. Szabo, L. Stolz, R. Granow, Curr. Opin. Struct. Biol. 5 (1995) 699–705.
[36] L.C. Feldman, J.W. Mayer, Fundamentals of Surface and Thin Film Analysis, PTR Prentice Hall, Englewood Cliffs, 1986.
[37] M.D. Ward, D.A. Buttry, Science 249 (1990) 1000–1007.
[38] R. Schumacher, Angew. Chem. Int. Ed. Engl. 29 (1990) 329–343.
[39] C.D. Bain, J. Evall, G.M. Whitesides, J. Am. Chem. Soc. 111 (1989) 7155–7164.
[40] G.B. Sigal, C. Bamdad, A. Barberis, J. Strominger, G.M. Whitesides, Anal. Chem. 68 (1996) 490–497.
[41] M. Mrksich, G.B. Sigal, G.M. Whitesides, Langmuir 11 (1995) 4383–4385.
[42] E. Delamarche, A. Bernard, H. Schmid, B. Michel, H. Biebuyck, Science 276 (1997) 779–781.
[43] E. Delamarche, A. Bernard, H. Schmid, A. Bietsch, B. Michel, H. Biebuyck, J. Am. Chem. Soc. 120 (1998) 500–508.
[44] M. Mrksich, L.E. Dike, J. Tien, D.E. Ingber, G.M. Whitesides, Exp. Cell Res. 235 (1997) 305–313.
[45] G.M. Whitesides, P.E. Laibinis, Langmuir 6 (1990) 87–96.
[46] A. Ulman, An Introduction to Ultrathin Organic Films, Academic Press, Boston, 1991.
[47] R.G. Nuzzo, D.L. Allara, J. Am. Chem. Soc. 105 (1983) 4481.
[48] P. Fenter, A. Eberhardt, P. Eisenberger, Science 266 (1994) 1216–1218.

[49] P. Fenter, F. Schreiber, L. Berman, G. Scoles, P. Eisenberger, M. Bedzyk, J. Surf. Sci. 1998, (Submitted for publication).
[50] R.G. Nuzzo, F.A. Fusco, D.L. Allara, J. Am. Chem. Soc. 109 (1987) 2358.
[51] R.G. Nuzzo, B.R. Zegarski, L.H. Dubois, J. Am. Chem. Soc. 109 (1987) 733–740.
[52] R.G. Nuzzo, L.H. Dubois, D.L. Allara, J. Am. Chem. Soc. 112 (1990) 558–569.
[53] P.L. Laibinis, G.M. Whitesides, D.L. Allara, Y.-T. Tao, A.N. Parikh, R.G. Nuzzo, J. Am. Chem. Soc. 113 (1991) 7152–7167.
[54] M.J. Tarlov, D.R.F. Burgess, G. Gillen, J. Am. Chem. Soc. 115 (1993) 5305–5306.
[55] Y.Z. Li, J.Y. Huang, R.T. McIver, J.C. Hemminger, J. Am. Chem. Soc. 114 (1992) 2428–2432.
[56] J.Y. Huang, J.C. Hemminger, J. Am. Chem. Soc. 115 (1993) 3342–3343.
[57] Y. Zhang, R.H. Terrill, T.A. Tanzer, P.W. Bohn, J. Am. Chem. Soc. 120 (1998) 2654–2655.
[58] M.H. Schoenfisch, J.E. Pemberton, J. Am. Chem. Soc. 120 (1998) 4502–4513.
[59] L.H. Dubois, B.R. Zegarski, R.G. Nuzzo, J. Vac. Sci. Technol. A5 (1987) 634.
[60] D.E. Riederer, R. Chatterjee, S.W. Rosencrance, Z. Postawa, T.D. Dunbar, D.L. Allara, N. Winograd, J. Am. Chem. Soc. 119 (1997) 8089–8094.
[61] P. DiMilla, J.P. Folkers, H.A. Biebuyck, R. Harter, G. Lopez, G.M. Whitesides, J. Am. Chem. Soc. 116 (1994) 2225–2226.
[62] N.B. Larsen, H. Biebuyck, E. Delamarche, B. Michel, J. Am. Chem. Soc. 119 (1997) 3017–3026.
[63] P.E. Laibinis, C.D. Bain, R.G. Nuzzo, G.M. Whitesides, J. Phys. Chem. 99 (1995) 7663–7676.
[64] L. Yan, C. Marzolin, A. Terfort, G.M. Whitesides, Langmuir 13 (1997) 6704–6712.
[65] L. Yan, X.-M. Zhao, G.M. Whitesides, J. Am. Chem. Soc. 120 (1998) 6179–6180.
[66] J. Lahiri, L. Isaacs, J. Tien, G.M. Whitesides, Anal. Chem. 1998, (In press).
[67] J. Lahiri, E. Ostuni, G.M. Whitesides, Langmuir 1998, (In press).
[68] R.C. Horton, T.M. Herne, D.C. Myles, J. Am. Chem. Soc. 119 (1997) 12980–12981.
[69] R.V. Duevel, R.M. Corn, Anal. Chem. 64 (1992) 337–342.
[70] S. Pan, D.G. Castner, B.D. Ratner, Langmuir 14 (1998) 3545–3550.
[71] L. Bertilsson, B. Liedberg, Langmuir 9 (1993) 141–149.
[72] C.D. Hodneland, M. Mrksich, Langmuir 13 (1997) 6001–6003.
[73] J.D. Swalen, D.L. Allara, J.D. Andrade, et al., Langmuir 3 (1987) 932–950.
[74] C.D. Bain, G.M. Whitesides, Angew. Chem. Int. Ed. Engl. 28 (1989) 506–516.
[75] L.H. Dubois, B.R. Zegarski, R.G. Nuzzo, J. Am. Chem. Soc. 112 (1990) 570–579.
[76] L. Strong, G.M. Whitesides, Langmuir 4 (1988) 546–558.
[77] C.E.D. Chidsey, G.-Y. Liu, P. Rowntree, G. Scoles, J. Chem. Phys. 91 (1989) 4421–4423.
[78] L.H. Dubois, B.R. Zegarski, R.G. Nuzzo, Langmuir 2 (1986) 412.
[79] M.D. Porter, T.B. Bright, D.L. Allara, C.E.D. Chidsey, J. Am. Chem. Soc. 109 (1987) 3559–3568.
[80] C.D. Bain, G.M. Whitesides, J. Am. Chem. Soc. 110 (1988) 6560–6561.
[81] P.E. Laibinis, R.G. Nuzzo, G.M. Whitesides, J. Phys. Chem. 96 (1992) 5097–5105.
[82] C.D. Bain, G.M. Whitesides, Science 240 (1988) 62–63.
[83] C.D. Bain, H.A. Biebuyck, G.M. Whitesides, Langmuir 5 (1989) 723–727.
[84] E.B. Troughton, C.D. Bain, G.M. Whitesides, R.G. Nuzzo, D.L. Allara, M.D. Porter, Langmuir 4 (1988) 365–385.
[85] M. Mrksich, G.M. Whitesides, in: J.M. Harris, S. Zalipsky (Eds.), Poly(ethylene glycol) Chemistry and Biological Applications, American Chemical Society, Washington, DC, 1997, pp. 361–373.
[86] S.I. Jeon, J.D. Andrade, J. Colloid Interface Sci. 142 (1991) 159–165.
[87] S.I. Jeon, L.H. Lee, J.D. Andrade, P.G. de Gennes, J. Colloid Interface Sci. 142 (1991) 149–158.
[88] T. McPherson, A. Kidane, I. Szleifer, K. Park, Langmuir 14 (1998) 176–186.
[89] D. Chin, G.M. Whitesides, Unpublished results.
[90] R.L.C. Wang, H.J. Kreuzer, M. Grunze, J. Phys. Chem. B. 101 (1997) 9767–9773.
[91] P. Harder, M. Grunze, R. Dahint, G.M. Whitesides, P.E. Laibinis, J. Phys. Chem. 102 (1998) 426–436.
[92] J.M. Harris, S. Zalipsky, Poly(ethylene glycol). Chemistry and Biological Applications, American Chemical Society, Washington, DC, 1997.
[93] L. Deng, M. Mrksich, G.M. Whitesides, J. Am. Chem. Soc. 118 (1996) 5136–5137.
[94] R.G. Chapman, L. Yan, S. Takayama, R.E. Holmlin, G.M. Whitesides, Unpublished results.
[95] M. Malmqvist, Nature 361 (1993) 186–187.
[96] H. Sota, Y. Hasegawa, M. Iwakura, Anal. Chem. 70 (1998) 2019–2024.
[97] C.E. Jordan, B.L. Frey, S. Kornguth, R.M. Corn, Langmuir 10 (1994) 3642–3648.
[98] K.A. Peterlinz, R. Georgiadis, Optics Comm. 130 (1996) 260–266.
[99] S. Lofas, M. Malmqvist, I. Ronnberg, E. Stenberg, B. Liedberg, I. Lundstrom, Sensors Actuators B 5 (1991) 79–84.
[100] U. Jonsson, M. Malmqvist, Advances in Biosensors, JAI Press, London, 1992, pp. 291–336.
[101] P. Schuck, Curr. Opin. Biotechnol. 8 (1997) 498–502.
[102] P. Schuck, Biophys. J. 70 (1996) 1230–1249.
[103] R.M.A. Azzam, N.M. Bashara, Ellipsometry and Polarized Light, North Holland, Amsterdam, 1977.
[104] C.F. Mandenius, S. Welin, B. Danielsson, I. Lundstrom, K. Mosbach, Anal. Biochem. 137 (1984) 106–114.

[105] H. Schonherr, J.F.B. Kremer, S. Kumar, et al., J. Am. Chem. Soc. 118 (1996) 13051–13057.
[106] F. Hook, M. Rodahl, B. Kasemo, P. Brzezinski, Proc. Natl. Acad. Sci. USA 95 (1998) 12271–12276.
[107] C.A. Keller, B. Kasemo, Biophys. J. 75 (1998) 1397–1402.
[108] G.S. Calabrese, H. Wohltjen, M.K. Roy, Anal. Chem. 59 (1987) 833–837.
[109] R. Dahint, M. Grunze, F. Josse, J. Renken, Anal. Chem. 66 (1994) 2888–2892.
[110] K.K. Chittur, D.J. Fink, R.I. Leininger, T.B. Hutson, J. Colloid Interface Sci. 1986, 111.
[111] S.-S. Cheng, K.K. Chittur, C.N. Sukenik, L.A. Culp, K. Lewandowska, J. Colloid Interface Sci. 162 (1994) 135–143.
[112] H.H. Bauer, M. Muller, J. Goette, H.P. Merkle, U.P. Fringeli, Biochemistry 33 (1994) 12276–12282.
[113] M. Muller, R. Buchet, U.P. Fringeli, J. Phys. Chem. 100 (1996) 10810–10825.
[114] M. Liley, T.A. Keller, C. Duschl, H. Vogel, Langmuir 13 (1997) 4190–4192.
[115] S.R. Wasserman, G.M. Whitesides, I.M. Tidswell, B.M. Ocko, P.S. Pershan, J.D. Axe, J. Am. Chem. Soc. 111 (1989) 5852–5861.
[116] I.M. Tidswell, B.M. Ocko, P.S. Pershan, S.R. Wasserman, G.M. Whitesides, J.D. Axe, Phys. Rev. B 41 (1990) 1111–1128.
[117] S. Petrash, N.B. Sheller, W. Dando, M.D. Foster, Langmuir 13 (1997) 1881–1883.
[118] Y. Xia, E. Kim, X.-M. Zhao, J.A. Rogers, M. Prentiss, G.M. Whitesides, Science 273 (1996) 347–349.
[119] D. Qin, Y. Xia, G.M. Whitesides, Adv. Mater. 8 (1996) 917–919.
[120] R. Jackman, J. Wilbur, G.M. Whitesides, Science 269 (1995) 664–666.
[121] K.A. Peterlinz, R. Georgiadis, Langmuir 12 (1996) 4731–4740.
[122] E. Kim, Y. Xia, G.M. Whitesides, Nature 376 (1995) 581–584.
[123] E. Kim, G.M. Whitesides, J. Phys. Chem. 101 (1997) 855–863.
[124] H.A. Biebuyck, G.M. Whitesides, Langmuir 10 (1994) 4581–4587.
[125] M.K. Chaudhury, G.M. Whitesides, Langmuir 7 (1991) 1013–1025.
[126] H.P. Jennissen, J. Chromatogr. 159 (1978) 71–83.
[127] M. Malmsten, B. Lassen, J. Colloid Interface Sci. 166 (1994) 490–498.
[128] M. Malmsten, J. Colloid Interface Sci. 172 (1995) 106–115.
[129] E. Ostuni, C.S. Roberts, M. Mrksich, G.M. Whitesides, Unpublished results.
[130] T. Arai, W. Norde, Colloids Surf. 51 (1990) 1–15.
[131] D. Asthagiri, A.M. Lenhoff, Langmuir 13 (1997) 6761–6768.
[132] C.M. Roth, A.M. Lenhoff, Langmuir 9 (1993) 962–972.
[133] M.A.C. Stuart, G.J. Fleer, J. Lyklema, W. Norde, J.M.H.M. Scheutjens, Adv. Colloid Interface Sci. 34 (1991) 477–535.
[134] J. Gao, M. Mammen, G.M. Whitesides, Science 272 (1996) 535–537.
[135] M. Mammen, I.J. Colton, J. Carbeck, R. Bradley, G.M. Whitesides, Anal. Chem. 69 (1997) 2165–2170.
[136] J.L. Robeson, R.D. Tilton, Langmuir 12 (1996) 6104–6113.
[137] M. Wahlgren, T. Arnebrandt, Langmuir 13 (1997) 8–13.
[138] V. Yaminsky, C. Jones, F. Yaminsky, B.W. Ninhan, Langmuir 12 (1996) 3531–3535.
[139] J.R. Rahn, K.H. Yeap, S.W. Barton, R.B. Hallock, Langmuir 1998, (Submitted for publication).
[140] B.L. Frey, C.E. Jordan, S. Kornguth, R.M. Corn, Anal. Chem. 67 (1995) 4452–4457.
[141] C.E. Jordan, A.G. Frutos, A.J. Thiel, R.M. Corn, Anal. Chem. 69 (1997) 4939–4947.
[142] C.E. Jordan, R.M. Corn, Anal. Chem. 69 (1997) 1449–1456.
[143] A.J. Thiel, A.G. Frutos, C.E. Jordan, R.M. Corn, L.M. Smith, Anal. Chem. 69 (1997) 4948–4956.
[144] P.A. Boriack, D.W. Christianson, J. Kingery-Wood, G.M. Whitesides, J. Med. Chem. 38 (1995) 2286–2291.
[145] J. Lahiri, L. Isaacs, B. Grzybovski, G.M. Whitesides, Langmuir 1998, (Submitted for publication).
[146] L. Yan, J. Rao, G.M. Whitesides, J. Am. Chem. Soc. 1997, (Submitted for publication).
[147] J. Rao, G.M. Whitesides, J. Am. Chem. Soc. 119 (1997) 10286–10290.
[148] H.G. Hong, M. Jiang, S.G. Sligar, P.W. Bohn, Langmuir 10 (1994) 153–158.
[149] T.M. Herne, M.J. Tarlov, J. Am. Chem. Soc. 119 (1997) 8916–8920.
[150] B. Alberts, D. Bray, A. Johnson, J. Lewis, M. Raff, K. Roberts, P. Walter, Essential Cell Biology. An Introduction to the Molecular Biology of the Cell, 1st ed., Garland Publishing, New York, 1998, pp. 481–512.
[151] C.H. Damsky, Z. Werb, Curr. Opin. Cell Biol. 4 (1992) 772–781.
[152] M.H. Ginsberg, X. Du, E.F. Plow, Curr. Opin. Cell Biol. 4 (1992) 766–771.
[153] D.E. Ingber, Curr. Opin. Cell Biol. 3 (1991) 841–848.
[154] D.A. Borkholder, J. Bao, N.I. Maluf, E.R. Perl, G.T.A. Kovacs, J. Neurosci. Methods 77 (1997) 61–66.
[155] D.A. Stenger, C.J. Pike, J.J. Hickman, C.W. Cotman, Brain Res. 630 (1993) 136–147.
[156] J.H.J. Georger, D.A. Stenger, A.S. Rudolph, J.J. Hickman, C.S. Dulcey, T.L. Fare, Thin Solid Films 210 (1992) 716–719.
[157] J.J. Hickman, S.K. Bhatia, J.N. Quong, P. Shoen, D.A. Stenger, C.J. Pike, C.W. Cotman, J. Vac. Sci. Technol. A 12 (1994) 607–616.
[158] E. Ostuni, C.S. Chen, D.E. Ingber, G.M. Whitesides, Unpublished results.
[159] A.J. You, R.J. Jackman, G.M. Whitesides, S.L. Schreiber, Chem. Biol. 4 (1997) 969–975.
[160] H.M. McConnell, J.C. Owicki, J.W. Parce, D.L. Miller, G.T. Baxter, H.G. Wada, S. Pitchford, Science 257 (1992) 1906–1912.

[161] J.W. Parce, J.C. Owicki, K.M. Kercso, et al., Science 246 (1989) 243–247.
[162] D.L. Miller, J.C. Olson, J.W. Parce, J.C. Owicki, Biophys. J. 64 (1993) 813–823.
[163] G.T. Baxter, D.L. Miller, R.C. Kuo, G. Wada, J.C. Owicki, Biochemistry 31 (1992) 10950–10954.
[164] D.A. Stenger, J.H. Georger, C.S. Dulcey, et al., J. Am. Chem. Soc. 114 (1992) 8435–8442.
[165] B.J. Spargo, M.A. Testoff, T.B. Nielsen, D.A. Stenger, J.J. Hickman, A.S. Rudolph, Proc. Natl. Acad. Sci. USA 91 (1994) 11070–11074.
[166] S.N. Bhatia, M.L. Yarmush, M. Toner, J. Biomed. Mater. Res. 34 (1997) 189–199.
[167] M. Matsuzawa, S. Tokumitsu, W. Knoll, H. Sasabe, Langmuir 14 (1998) 5133–5138.
[168] M.S. Ravenscroft, K.E. Bateman, K.M. Shaffer, et al., J. Am. Chem. Soc. 120 (1998) 12169–12177.
[169] C.S. Dulcey, J.H. Georger, V. Krauthamer, D.A. Stenger, T.L. Fare, J.M. Calvert, Science 252 (1991) 551–554.
[170] L.F. Rozsnyai, D.R. Benson, S.P.A. Fodor, P.G. Schultz, Angew. Chem. lnt. Ed. Engl. 31 (1992) 759–761.
[171] J. Fujii, E. Ostuni, G.M. Whitesides, L.G. Griffith, Unpublished results.
[172] M. Bailly, L. Yan, G.M. Whitesides, J.S. Condeelis, J.E. Segall, Exp. Cell Res. 241 (1998) 285–299.
[173] C.S. Chen, M. Mrksich, S. Huang, G.M. Whitesides, D.E. Ingber, Biotech. Prog. 1998, (In press).
[174] C.S. Chen, E. Ostuni, G.M. Whitesides, D.E. Ingber, in: C. Streuli, M. Grant (Eds.), Methods in Molecular Biology: Extracellular Matrix, Humana Press, Totowa, NJ, 1998, (Submitted for publication).
[175] A. Bernard, E. Delamarche, H. Schmid, B. Michel, H.R. Bosshard, H. Biebuyck, Langmuir 14 (1998) 2225–2229.
[176] C.D. James, R.C. Davis, L. Kam, H.G. Craighead, M. Isaacson, J.N. Turner, W. Shain, Langmuir 14 (1998) 741–744.
[177] M.D. Pierschbacher, E. Ruoslahti, Nature 309 (1984) 30–33.
[178] E. Ruoslahti, M.D. Pierschbacher, Science 238 (1987) 491–497.
[179] J.A. Massia, S.P. Hubbell, J. Cell Biol. 114 (1991) 1089–1100.
[180] D.E. Ingber, J. Folkman, J. Cell Biol. 109 (1989) 317–330.
[181] D.E. Ingber, Proc. Natl. Acad. Sci. USA 87 (1990) 3579–3583.
[182] J.R. Sims, S. Karp, D.E. Ingber, J. Cell Sci. 103 (1992) 1215–1222.
[183] L. Chou, J.D. Firth, V.-J. Uitto, D.M. Brunette, J. Cell Sci. 108 (1995) 1563–1573.
[184] P. Clark, P. Connolly, A.S.G. Curtis, J.A.T. Dow, C.D.W. Wilkinson, Development 108 (1990) 635–644.
[185] H.C. Hoch, R.C. Staples, B. Whitehead, J. Comeau, E.D. Wolf, Nature 235 (1987) 1659–1662.
[186] J. Meyle, K. Gultig, M. Brich, H. Hammerle, W. Nisch, J. Mater. Sci. Mater. Med. 5 (1994) 463–466.
[187] J. Charnley, Acrylic Cement in Orthopaedic Surgery, The Williams and Wilkins, Co, Baltimore, 1970.
[188] Y. Kimura, in: T. Tsuruta, T. Hayashi, K. Kataoka, K. Ishihara, Y. Kimura (Eds.), Biomedical Applications of Polymeric Materials, CRC Press, Boca Raton, Florida, 1993, pp. 163–189.
[189] N.M.K. Lamba, K.A. Woodhouse, S.L. Cooper, Polyurethanes in Biomedical Applications, CRC Press LLC, Boca Raton, Florida, 1998.
[190] T. Nishimura, in: T. Tsuruta, T. Hayashi, K. Kataoka, K. Ishihara, Y. Kimura (Eds.), Biomedical Applications of Polymeric Materials, CRC Press, Boca Raton, Florida, 1993.
[191] B.D. Ratner, A.S. Hoffmann, F.J. Schoen, J.E. Lemons, Biomaterials Science. An Introduction to Materials in Medicine, Academic Press, New York, 1996, pp. 1–131.
[192] M.F. Refojo, in: Kirk/Othmer Encyclopedia of Chemical Technology, Wiley Interscience, New York, NY, 1979, pp. 720–742.
[193] J.F. Kang, R. Jordan, A. Ulman, Langmuir 14 (1998) 3983–3985.
[194] C.D. Bain, E.B. Troughton, Y.-T. Tao, J. Evall, G.M. Whitesides, R.G. Nuzzo, J. Am. Chem. Soc. 111 (1989) 321–335.
[195] I. Engquist, M. Lestelius, B. Liedberg, Langmuir 13 (1997) 4003–4012.
[196] P.W. Taylor, R.W. King, A.S.V. Burgen, Biochemistry 9 (1970) 2638–2645.