Using self-assembled monolayers to understand the biomaterials interface

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Self-assembled monolayers of alkanethiolates on gold and of supported lipids are structurally well defined surfaces that have been important in understanding the relationships between the structure of a material and the interaction of proteins with the material. The synthetic flexibility available with these model surfaces makes it possible to design surfaces that resist the adsorption of protein, and also surfaces to which proteins can be covalently or reversibly immobilized. The past several years have seen advances in techniques that can pattern and control the topography of surfaces, these methods can create tailored substrates for attached cell culture.

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Abbreviations

CA	carbonic anhydrase
μCP	microcontact printing
NTA	nitrilotriacetic acid
PEG	Poly(ethylene glycol)
SAM	self-assembled monolayer
SPR	surface plasmon resonance spectroscopy

Introduction

Examples of interfaces that join biological media and man-made materials are prevalent and include the use of contact lenses, dental fillings, and implantable devices ranging from pacemakers to drug release polymers for passive birth control. With few exceptions, the materials used in these and other medical and biotechnological applications (Table I) were not developed for these purposes [1•,2•]. It is reasonable to expect that these materials are not optimized and that the rational design—at the molecular scale—of new materials will produce biomaterials that are better suited for these applications and for many others not yet realized.

The past several years have seen significant advances in the development of model surfaces that have well defined and tailorable structures, and in the development of analytical methods that can characterize the structures and interactions of these surfaces with biological components. Together with an improved understanding of the mechanisms of biological responses to materials, these advances now provide a practical framework with which to understand and control, at the molecular scale, the interactions between the materials and biological components. Much of this work has focused on protein adsorption to materials and on strategies to create interfaces that present proteins in controlled environments. This understanding in turn provides a basis for the design of materials that control the attachment and behavior of mammalian cells. This short review highlights a selection of recent work that has used well defined interfaces to understand the relationships between the structure of a material and its interactions with proteins and cells, and to design surfaces that have designated properties.

Table 1

Materials used in medical applications.

Material	Application
Pyrolytic carbon	Heart valves
Titanium alloys	Joint replacements
Poly(urethane)	Artifical heart
Poly(propylene)	Tendon prostheses
Polyesters	Sutures and drug delivery
Poly(ethylene)	Catheters
Poly(hydroxyethyl methacrylate)	Contact lenses

The event that almost always follows the placement of a material in contact with a biological fluid is the adsorption of protein to the nonnatural surface. All subsequent biological responses to the material, including antigenic response, the attachment and growth of cells, and thrombosis, depend critically on the layer of adsorbed protein. An understanding of the properties of biomaterials must start with a description of the orientation and conformation of protein, or mixture of proteins, that adsorbs to the nonnatural surface. In real systems, this description is complicated by the heterogeneity in the layer due to many different conformations of adsorbed protein, to changes in the conformation (denaturation) of protein and to the exchange of adsorbed protein with soluble protein. Mechanisms of protein adsorption have received much attention from investigators with a broad range of backgrounds, because of their central importance in the biomaterials interface. Several excellent reviews and monographs are available that discuss this work [3–6].

Analytical methods

Studies of protein adsorption require analytical techniques that can measure as little as $10\,pg\,mm^{-2}$ of protein adsorbed to a surface; techniques that do not require the protein to be labeled with a chromophore to enhance sensitivity are preferred. Because adsorption is usually irreversible (on practical time scales) it is important that the technique also measures adsorption *in situ* and in real time to provide kinetic information. Ellipsometry

is an excellent technique that measures the average amount of protein adsorbed to an interface, but it is less convenient for *in situ* studies of adsorption [7]. Methods that use piezoelectric devices, including the quartz crystal microbalance [8], the surface acoustic wave device [9] and acoustic plate mode sensor [10], measure adsorption *in situ*, in real time and with good sensitivity but have the principle disadvantage that changes in solution conditions (flow rate, temperature, ion composition) can interfere with the signal. Surface plasmon resonance spectroscopy (SPR) is an optical technique that measures changes in the index of refraction of the interfacial region and is perhaps the best suited technique for studying adsorption. SPR is especially well suited for studies of adsorption on self-assembled monolayers of alkanethiolates [11•] because it uses thin films of gold. The availability of a commercial instrument now makes this technique accessible to many researchers. The principle disadvantage with all of these techniques, of course, is that they do not provide structural information about adsorbed proteins.

Structural characterization of adsorbed protein

There exist few analytical methods—and none with the power to reveal structure comparable to X-ray crystallography or multi-dimensional nuclear magnetic resonance spectroscopy—that can characterize the conformation and orientation of proteins adsorbed to surfaces. For the special cases involving two-dimensional ordered arrays of protein, electron diffraction can provide structural information. Kornberg and coworkers [12] have examined the structure of crystals of streptavidin attached to a lipid layer presenting biotin groups. Rennie and coworkers [13] have used a related technique based on neutron reflection to determine the structure of β-casein adsorbed to hydrophobic alkylsiloxane monolayers. Neutron diffraction has even been used to characterize the orientation of carbohydrates attached to model membranes [14]. A technique that uses X-ray standing waves also provides direct structural information with near-atomic resolution for cases where the protein contains a heavy atom group [15].

Spectroscopic methods can provide important but qualitative information on the conformation of adsorbed proteins. Infrared spectroscopy in the amide region was used to follow loss in the secondary structure of lysozyme adsorbed to silica surfaces [16]. Techniques that use polarized spectroscopies can determine the average orientation of adsorbed proteins that contain an optical chromophore (most commonly the heme group) within the tertiary structure [17,18]. Scanning tunneling and atomic force microscopies provide direct images of proteins adsorbed to materials [19]. These techniques are still very limited in resolution, but they may prove useful in investigating the heterogeniety in proteins adsorbed to a surface [20].

A range of 'footprinting' techniques from molecular biology have been used to identify the regions of adsorbed protein that are accessible to solvent; this information in turn suggests the orientation of the protein on the surface. Oxygen radicals, for example, react with proteins to cleave the polypeptide backbone. If the cleavage reaction is nonspecific but is inhibited by the presence of the surface and neighboring proteins, only the exposed regions of the protein should react: subsequent analysis of the cleavage products by gel electrophoresis reveals the sites of cleavage [21]. Walker and Grant [22] have used this strategy to characterize the conformation of DNA strands adsorbed to latex particles. In a related, but nondestructive method, Smith and coworkers [23] inferred the orientation of membrane proteins by measuring the exchange of its acidic protons with deuterium ion in solution. These methods provide interpretable data only in cases where proteins are uniformly oriented at the surface.

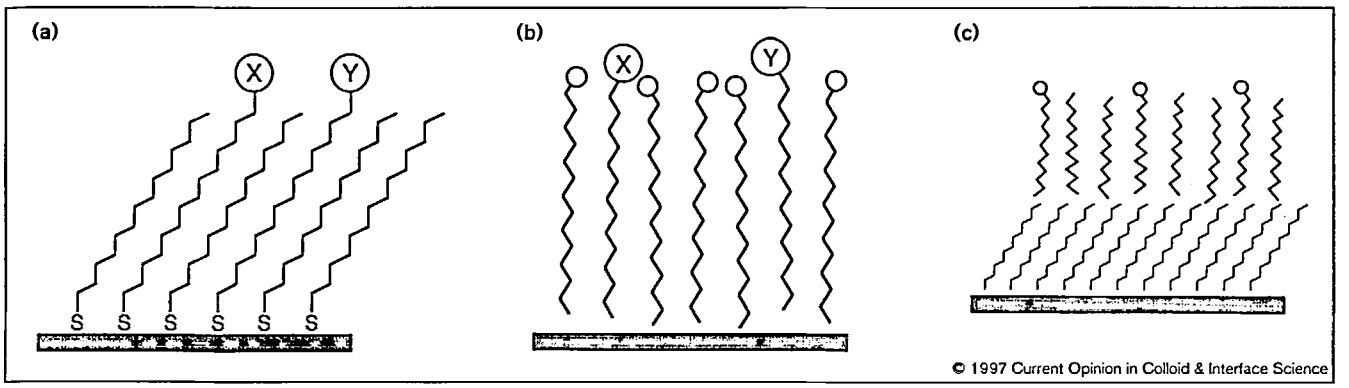
Model organic interfaces

Perhaps the most exciting advances in biomaterials research have involved the development of model organic surfaces that provide a convenient synthetic methodology for the preparation of structurally well defined interfaces (Fig. 1) (for reviews, see [24•,25,26]). Self-assembled monolayers (SAMs) formed upon the adsorption of alkanethiols on gold, and to a lesser extent alkyltrichlorosilanes on hydroxylated surfaces, are structurally the best-ordered interfaces. SAMs of alkanethiolates on gold are easily prepared by immersing a clean film of gold into a solution of terminally-substituted alkanethiols. The molecules assemble on the surface to give a dense-packed film that presents the terminal substituents at the interface. The structure of the interface is easily manipulated through synthesis of the precursor alkanethiol.

Langmuir–Blodgett films are formed by the assembly of amphiphilic molecules on solid or liquid interfaces. The structures of these interfaces can also be controlled through synthesis of the precursor amphiphiles (Fig. 1b). These lipid layers differ in many respects from monolayers that are attached through bonds to a solid support. They are more difficult to prepare and are usually less stable than the other monolayers. The molecules are free to diffuse within the layer and often separate into domains of single constituents. They have the principal advantage, though, that they are excellent substrates for immobilizing membrane-confined proteins.

These interfaces have been instrumental in understanding the relationships between the structure of an interface and its properties in a variety of areas, and particularly in biointerfacial science [26]. The next section discusses the design of well defined interfaces that resist the adsorption of protein, and of interfaces that use tailored chemistries to control the immobilization of protein.

Figure 1



Well defined organic monolayers that are useful for studies of the biomaterials interface include **(a)** long chain alkanethiolates assembled on the surface of gold and **(b)** amphiphilic molecules assembled on solid or liquid interfaces. Both classes of interfaces allow a variety of groups (X,Y) to be presented at the interface by incorporating the functionalized precursors. **(c)** The two classes of interfaces can be combined when monolayers of alkanethiolates are used as a support for the lipid layer.

Surfaces that resist the adsorption of protein

A central goal in biomaterials research is the identification and design of new inert materials that resist the nonspecific adsorption of protein. Poly(ethylene glycol) (PEG) is the most common inert biomaterial in use, and a variety of strategies for tailoring the surfaces of materials with PEG have been developed [27]. Prime and Whitesides [28] showed that SAMs presenting short oligomers of the ethylene glycol group $[-S(CH_2)_{11}(OCH_2CH_2)_nOH$, n = 2–7) are also highly effective at resisting the adsorption of protein [28]. This work demonstrated that even thin, densely packed layers of PEG are effective at preventing adsorption, and it provides a tractable system for the identification of other groups having this property. Deng *et al.* reasoned that oligomers of the propylene sulfoxide group, like those of the ethylene glycol group, were hydrophilic and conformationally flexible in aqueous environments. They prepared SAMs presenting tri(propylene sulfoxide) groups and found that these interfaces were also highly effective at resisting the adsorption of most proteins [29]. This study demonstrates the role of well defined model surfaces in developing new materials having designated properties, and in this case suggests a new polymer for development as a biomaterial.

Immobilization of proteins to surfaces

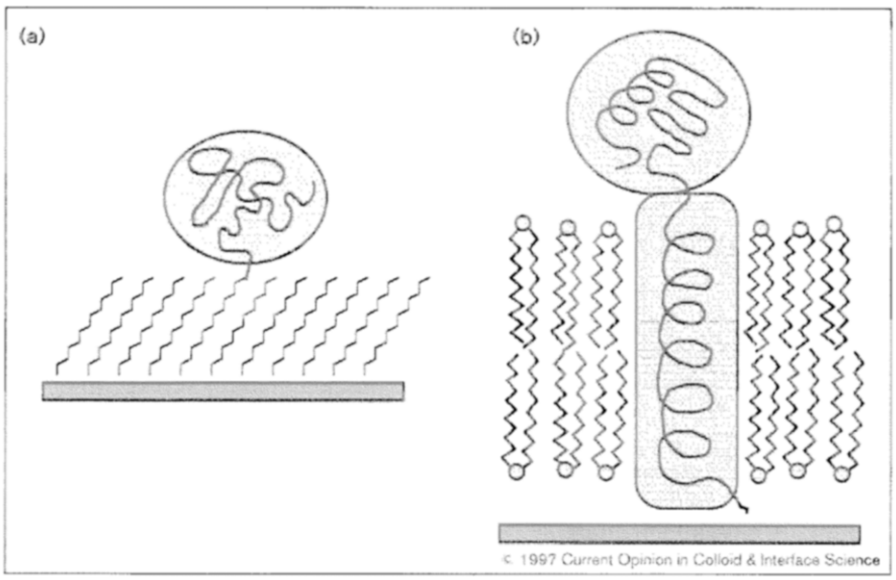
An extensive body of work now makes it routine to immobilize proteins to interfaces [30–32]. The methods used range from those that simply adsorb proteins to surfaces, to methods that covalently link proteins to functional groups of the interface. Methods that react a functional group of the protein with a functional group of the surface (one common example is the condensation of lysine ε-amino groups of a protein with carboxylic acids of a surface) often provide a heterogenous family of attached proteins due to reaction with one of several functional groups on the protein. The current challenge is to control explicitly the conformation, orientation and density of attached proteins (Fig. 2). Sligar and his coworkers [33•] have used genetic engineering to create a mutant of myoglobin that contains a single cysteine residue. This protein was linked to an alkylsiloxane monolayer presenting thiol groups via a disulfide bond to give a uniformly oriented layer of myoglobin. Michel and coworkers [34•] described a different strategy, based on a photochemically active group, for immobilizing proteins. When combined, these, and related methods that use selective chemistries, will allow the immobilization of several different proteins to a single substrate, with independent control over the density and environment of each protein.

Many proteins present on the cell surface reside within the bilayer membrane and denature when removed from the membrane. It is consequently difficult to isolate and immobilize these proteins to SAMs. Several groups have instead used lipid bilayers to present these proteins (Fig. 2). Incorporation of a lipid-tagged antibody into a phospholipid monolayer gave a surface that bound the respective antigen [35]. These layers do have the disadvantages of limited stability and of free diffusion of proteins within the layer to form domains. Ringsdorf and coworkers [36•] have described a strategy wherein the proteins and lipid molecules may be linked to the underlying matrix. This arrangement places the protein in the favorable membrane-like environment, but reduces lateral diffusion of proteins and increases stability of the monolayer [36•].

Bio-specific adsorption of protein

Many applications require interfaces that bind specific proteins reversibly. The challenge is to design surfaces that present ligands for specific recognition of receptors, but at the same time resist the nonspecific adsorption of other proteins. One approach uses a supported monolayer of functionalized lipids to create an interface that mimics

Figure 2



Immobilization of proteins to surfaces. **(a)** A range of chemistries can be used to immobilize proteins to self-assembled monolayers. **(b)** Lipid bilayers are uniquely suited for presenting membrane-spanning proteins that otherwise denature when removed from the hydrophobic environment.

the cell surface. Stevens and coworkers [37•] used a cross-linked layer of lipids presenting carbohydrate ligands to create an optical sensor for toxins. This and other work has used self-assembled monolayers as a structurally well defined support for the ligand-bearing lipids [38]. Alternatively, the ligands may be attached directly to the monolayer, provided that the surface still resists nonspecific adsorption. Grunwell, Whitesides and I [39•] have used monolayers presenting oligo(ethylene glycol) groups and benzenesulfonamide groups—which are potent inhibitors of the enzyme carbonic anhydrase (CA)—to create substrates that adsorb CA. SPR spectroscopy showed that the surfaces were indeed specific for this protein and did not bind other proteins. Surfaces that present DNA oligonucleotides constitute an important class of bio-specific interfaces, and have motivated the development of many strategies to conjugate these oligomers to surfaces [40].

Whitesides and coworkers [41] have described a strategy based on chemical specificity to attach proteins to SAMs. Motivated by the well known association of oligo(histidine) peptides with complexes of nickel(II), his group prepared SAMs presenting nitrilotriacetic acid (NTA) chelates of Ni(II) and tri(ethylene glycol) groups, and demonstrated the specific attachment of His-tagged proteins [41]. The layer of protein was stable, but could be removed by the addition of competing imidazole ligands.

Attachment of cells to interfaces

Well defined surface chemistries can be combined with patterning techniques to create substrates that control the attachment of cells to designated regions. Much of this work employs a similar strategy and only differs primarily in the method used to pattern the substrate and the treatment of the surface that renders regions of the substrate inert to the attachment of cells. Photolithography, for example, was used to pattern alkylsiloxanes into regions presenting amino and perfluorinated groups [42]. Endothelial cells attached to, and spread only on the amino-terminated regions. The fluoro-terminated regions are not inert to protein adsorption, but likely adsorb proteins that do not permit cells to attach. Many methods use the adsorption of the protein serum albumin to surfaces to prevent cell attachment. A recent report by Bright and coworkers [43] shows that even this strategy can fail when the albumin is presented in a particular conformation that promotes the attachment of endothelial cells.

Whitesides and his group [44] have pioneered a nonlithographic technique that can pattern monolayers. Microcontact printing (μCP) uses an elastomeric stamp to pattern the formation of SAMs; μCP can be performed in an ordinary laboratory and can pattern monolayers at the micron length scale [44]. Contact printing was used to pattern monolayers of alkanethiolates on gold into regions terminated in methyl groups and oligo(ethylene glycol) groups. After allowing fibronectin to adsorb to the hydrophobic regions, hepatocyte cells attached and spread only on the hydrophobic regions [45]. This methodology has the distinction that inert regions of the monolayer are well defined and stable; the method does not require adsorption of serum albumin or other polymers to prevent the attachment of cells. In a recent report, Fuhr and coworkers [46•] showed that local electric fields created by alternating currents repel cells from the surface and prevent attachment. This method was used to pattern cell attachment on surfaces having interdigitated electrode arrays.

Cell attachment on contoured substrates

Additional techniques from microfabrication can be used to extend much of this work to nonplanar substrates [47•]. Chou *et al.* [48] fabricated substrates contoured into micron-sized grooves and found that the biosynthetic activities of attached fibroblasts were influenced by the topography of the substrate. Whitesides and coworkers [49] have used techniques related to μCP to prepare films of polyurethane contoured into grooves and ridges 25–50 μm in width. Evaporation of a thin, optically-transparent layer of gold on this film provided substrates whose properties could be tailored with SAMs. These substrates were used in turn to control the attachment of endothelial cells to either the grooves or ridges [50•].

Conclusions

Further work in biomaterials, for both fundamental studies and applications, will benefit from increasing the structural complexity of interfaces and from employing better analytical methods to characterize interfacial properties. A report by Massia and Hubbell [51], for example, showed that alkylsiloxanes that present peptides containing the Arg-Gly-Asp sequence supported the attachment and spreading of cells; these designed substrates eliminate the need for adsorbing matrix proteins to cell culture substrates and permit mechanistic studies of cell adhesion and spreading. Britland and McCaig [52] have used patterned substrates in combination with oriented electrical fields to affect the growth of nerve cells; this work provides new strategies to help understand the behavior of cells. I believe that electroactive and photoactive interfaces will become important in controlling interfacial properties [53].

Methods to prepare and characterize interfaces are now developed to the point where they are broadly useful for studies in biointerfacial science. SAMs of alkanethiolates on gold in particular offer wide flexibility in preparing structurally-complex interfaces. This review describes a number of examples that have used these preparative and analytical surface methodologies to understand and control the properties of materials in contact with proteins and cells. I believe that these examples illustrate just the beginning of biomaterials research and the use of designed interfaces, and that there remain countless opportunities in fundamental research and in applied materials.

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