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Review

Tailored substrates for studies of attached cell culture

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Abstract. Substrates for studies of the interactions of attached cells with extracellular matrix components are often prepared by allowing a protein to adsorb from solution onto a glass or polystyrene substrate. This method is simple and effective for many studies, but it can fail in cases that require rigorous control over the structure and composition of adsorbed protein. Self-assembled monolayers formed by the spontaneous ordering of terminally functionalized alkanethiols onto a gold substrate are a class of well-ordered substrates and provide a convenient method for tailoring substrates with ligands, proteins and other groups. Methods that can pattern the monolayers provide a general strategy to create substrates that control the size, shape and spacing of attached cells. This review illustrates recent work that has used these methods of surface chemistry to create tailored substrates for studies in cell biology.

Key words. Self-assembled monolayer; surface chemistry; biosurface; cell adhesion.

Introduction

The growth, differentiation and metabolism of cells are influenced by a multitude of signals present in the immediate environment. Among the most important signals are the ligands dissolved in the media and those present on the insoluble extracellular matrix surrounding cells. Studies of the effects of soluble signals on cellular behavior have utilized defined media and have been aided by synthetic methods that can prepare both natural and nonnatural peptides and ligands. Studies of the effects of immobilized ligands, by comparison, have been limited by a lack of convenient experimental methods for controlling the composition and structure of substrates for attached cell culture. The most common substrates – those prepared by allowing matrix proteins to adsorb to glass or polystyrene substrates – have the limitation that it is difficult to control and characterize the densities and structures of immobilized proteins.

This limitation has created a need for substrates that have several characteristics: (i) the adsorbed layer of protein is compositionally pure in that only select proteins, peptides and ligands are present; (ii) the presentation of immobilized proteins is homogeneous in that they are each adsorbed in a single orientation and conformation; (iii) the density of immobilized proteins can be controlled and measured; (iv) the immobilized proteins are stable and persistent in that the activity is not lost due to denaturation, exchange with proteins in the medium or degradation by cellular proteases. These substrates will find even wider application with the introduction of strategies that can change the pattern of ligands presented to a cell.

The recent development of synthetically flexible surface chemistries now makes possible the preparation of tailored substrates for many studies of the behaviour of attached cells. This review begins with a summary of the advantages and limitations of current methods for

preparing substrates that present adsorbed protein. The review then introduces methods from organic surface science that can prepare structurally well-defined substrates, and gives examples of the use of these substrates for studies involving attached cell culture. The discussion is then extended to include methods from microfabrication and lithography that can pattern the structure of substrates, and the use of these substrates to control the shapes, sizes and positions of attached cells. The review concludes with a survey of emerging techniques that can create substrates with dynamic control over structure and properties, and the utility of these substrates for studies of cell-substrate interactions.

Substrates with adsorbed protein

The most important method for preparing substrates for studies involving attached cell culture is to allow proteins or antibodies to adsorb from solution onto glass or plastic substrates. This method is experimentally convenient and general, and it provides substrates that in many respects resemble the in vivo environment of cells. Commercial sources of these matrix preparations and even precoated substrates are commonly available.

The limitations inherent in this method are all related to the complex mechanisms underlying protein adsorption (for reviews, see refs 1–3). There is an extensive literature in experimental studies of protein adsorption at the solid-liquid interface: many of these studies have not directly addressed the role of adsorbed protein in substrates for attached cell culture, but these studies do provide general guidelines that are relevant to understanding the preparation of substrates for cell culture. In solution, most proteins are folded into a single, discrete conformation. But on adsorption to a solid interface, a protein can assume a heterogeneous population of structures. Figure 1 summarizes the course of events that can follow the initial adsorption of protein (a). These events include lateral diffusion of protein (b) and denaturation of the protein (d) at the interface.

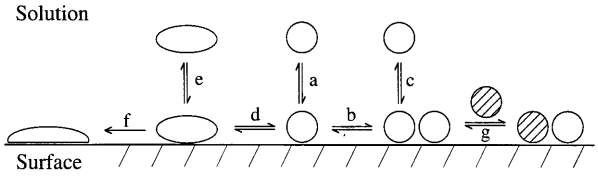


Figure 1. Scheme illustrating the complexities associated with the preparation of substrates by protein adsorption. Several events lead to a structurally heterogeneous layer of adsorbed protein: see text for an explanation.

Each of these different forms of protein can desorb from the interface (a, c, e), but usually the proteins remain irreversibly adsorbed to the interface (f), or they can exchange with soluble protein (g). Even in the near ideal case – that of a single, conformationally stable protein allowed to adsorb to a structurally homogeneous substrate – the resulting layer of adsorbed protein is often heterogeneous in structure. Because the biological activity of a protein is determined by its structure, the properties of these substrates are difficult to control.

The small amount of protein that is present in an adsorbed layer makes it extremely difficult to characterize the changes in structure that follow denaturation or reorientation at a substrate. While physical and spectroscopic techniques do not yet make routine the determination of molecular structure of proteins adsorbed at interfaces, several indirect strategies have been used to infer structural information. In studies of the enzyme ribonuclease A adsorbed to mica substrates, for example, Lee and Belfort showed that the activity of the enzyme increased over a period of 24 h [4]. The change in activity was ascribed to a change in the orientation of enzyme at the interface. Middlaugh and co-workers used a combination of calorimetry and fluorescence spectroscopy to show that, for several different proteins and substrates, adsorption destabilized the native structure and increased denaturation of proteins [5]. The adsorption of protein can also change dramatically with subtle changes in the structure of the protein. Ramsden and co-workers, for example, showed that the initial rates of adsorption onto a silicon oxide surface of two cytochrome $\beta 5$ fragments (that differed by switching two residues in the primary sequence) varied by over 10-fold [6].

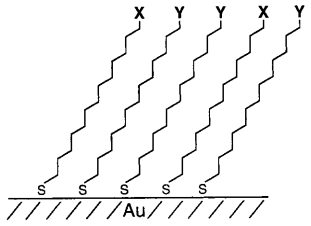
Changes in the conditions present during adsorption – including changes in ionic composition, temperature and pH – can also have dramatic effects on the structure of adsorbed protein. Tiberg and co-workers showed that the density of β-casein adsorbed to hydrophobic silica increased when the dissolved salt was changed from Na^+ to Mg^{+2} to Ca^{+2} [7]. The structure of the protein layer also depends on the concentration of protein in the solution from which it adsorbs. Studies of the adsorption of fibrinogen to a hydrophobic monolayer showed that the final density of protein increased by fivefold when the concentration of protein was increased from 3 to 280 μg/ml [8]. A kinetic analysis of these data was consistent with a mechanism that involved unfolding of the protein after adsorption. For low concentrations of protein, the initial rate for adsorption is slower and is followed by denaturation. For higher concentrations, the surface is rapidly saturated with protein, and denaturation is prevented. This effect makes it difficult to rigorously control the density of

adsorbed protein. For these reasons, matrix-coated substrates must be prepared under exactly the same conditions to insure that the structure of adsorbed protein – and hence the biological properties of the substrate – remain constant.

The ease and generality with which protein-coated substrates can be prepared make them an excellent choice for many studies of cell-matrix interactions. The purpose of the preceding section of the review was to emphasize the limitations of this methodology for those applications that require rigorous control over the structure of the substrate: for example those that require a quantitative analysis of the relationship between density of immobilized ligand and cellular structure. The use of structurally tailored substrates provides an alternate strategy that avoids these limitations. The next section introduces a class of structurally tailored substrates that allows rigorous control over the presentation of protein and ligands. These substrates have been important in a number of studies in biointerfacial science, and their utility is growing rapidly.

Structurally well defined substrates

This section will present an overview of the use of organic thin film substrates for attached cell culture. This section will focus on self-assembled monolayers of alkanethiolates on gold, because this class of monolayer is synthetically the most versatile (for reviews, see refs 9, 10]. Other classes of structurally well defined interfaces include crystals [11], metals [12] and supported layers of lipids [13]. While these interfaces make excellent substrates for many studies of protein adsorption and cell attachment, they have the primary limitations that the interfacial structure cannot be tailored easily through synthesis and they can have poor structural stability.



X, Y	Reference
Functional Groups	16
Synthetic Receptor	17
Ligands	18, 19
Peptides	20, 21
Proteins	22, 23
Oligonucleotides	24

Figure 2. Representation of the structure of a SAM of alkanethiolates on gold. The sulphur atoms coordinate to the gold and the *trans*-extended alkyl chains present the terminal groups (X, Y) at the interface. The table at the right gives examples (with references) of the variety of groups that have been attached to monolayers.

Self-assembled monolayers

Self-assembled monolayers (SAMs) of alkanethiolates on gold form upon the adsorption of long-chain alkanethiols, RSH [$R = X(CH_2)_n$, $n = 11–18$] from solution to a gold surface. For concentrations of alkanethiol near 1 mM in ethanol, the monolayer assembles in a period of 1 to 5 h. The structure of these monolayers is well established (fig. 2) [14, 15]. The sulphur atoms coordinate to the threefold sites of the gold(111) surface to give a close-packed array of alkyl chains. These chains are *trans*-extended and tilted approximately 30 °, and present the terminal functional group X at the surface; these exposed groups determine the properties of the interface. Even alkanethiols that are substituted with complex groups assemble into well-ordered monolayers that present these groups at the interface; alternatively, groups can be introduced onto the surface after the SAM is formed. Figure 2 gives examples of the range of groups that have been conjugated to SAMs. The properties of SAMs can be controlled further by formation of 'mixed' SAMs from solutions of two or more alkanethiols. SAMs on gold are stable in air or in contact with water for periods of months. The monolayers do undergo desorption at temperatures greater than 70 °C or when irradiated with ultraviolet (UV) light in the presence of oxygen. SAMs have sufficient stability in aqueous media for use in cell culture for periods of days. The primary disadvantage encountered in working with these monolayers is the requirement for gold-coated substrates. These substrates can be prepared by electron beam evaporation or sputtering of the metal, but they are not yet commercially available.

Monolayers that resist the adsorption of protein

The finding by Prime and Whitesides that monolayers presenting short oligomers of the ethylene glycol ($–OCH_2CH_2–$, EG) group are very effective at resisting the adsorption of protein was critical to making this surface chemistry broadly useful for biological applications [25, 26]. Monolayers presenting either the short tri(ethylene glycol) group or the longer hexa(ethylene glycol) group were completely resistant to the adsorption of protein. The degree to which these monolayers were inert could be estimated by diluting the ethylene glycol chains with a methyl-terminated alkanethiolate [$–S(CH_2)_{10}CH_3$]. Studies using surface plasmon resonance (SPR) – a technique that measures the adsorption of protein to interfaces in real time and in situ [27] – showed that SAMs wherein greater than 50% of the alkanethiolate chains present the glycol group resisted the adsorption of virtually all proteins under a range of solution conditions; they even prevented the adsorption of the 'sticky' protein fibrinogen [28]. The mechanisms by which these thin films resist adsorption are not yet

well understood, but the use of these monolayers as inert interfaces has been critical to a number of applications (see below) [29] .

Methods for patterning the formation of monolayers

Substrates that are patterned into regions that alternately support or resist the attachment and spreading of cells provide an effective method for examining the effects of cell shape and form on cellular behaviour. Several groups have demonstrated methods for preparing patterned substrates. These methods are all related in that they begin with a step that defines a pattern on the substrate and then selectively modify the surface to render selected regions inert. The most important methods for patterning interfaces rely on photolithography and microcontact printing.

Photolithography is a technique that was developed for fabricating microelectronics circuits. The method illuminates a substrate with UV light that is passed through a mask that has a pattern defined by transparent and opaque regions. Upon exposure to the light source, the substrate can be modified in a number of ways. For SAMs of alkanethiolates on gold, the UV light causes oxidative removal of the monolayer. The pattern of exposed gold that is created can then be modified with a monolayer presenting other groups [30]. Photolithography has been much more important for patterning monolayers of alkylsiloxanes on the surfaces of glass and silicon oxide. The common method starts with a silicon substrate coated with a thin layer of photoresist – a polymer that is degraded by UV light. Illumination of the substrate through mask, followed by a washing step, removes the polymer in exposed regions to reveal a silicon oxide surface. A monolayer can then be assembled on these regions [31]. The remaining photoresist is then removed by washing, and a different alkylsiloxane can be formed in the complementary regions. These techniques are very well developed. They do have the disadvantage, however, that the photolithographic equipment and a controlled environment facility make these techniques expensive, and substantially less convenient than microcontact printing.

Microcontact printing (μCP) uses a rubber stamp to print a patterned monolayer of alkanethiolates onto a gold substrate [32]. The procedure is illustrated in figure 3, and starts with the fabrication of a stamp using photolithography. A polished silicon wafer is coated with a thin layer of photoresist (a) and then exposed to intense UV light through a mask. Washing the substrate removes exposed regions of photoresist (b). The elastomeric stamp used in μCP is prepared by casting polydimethylsiloxane (PDMS) against the patterned

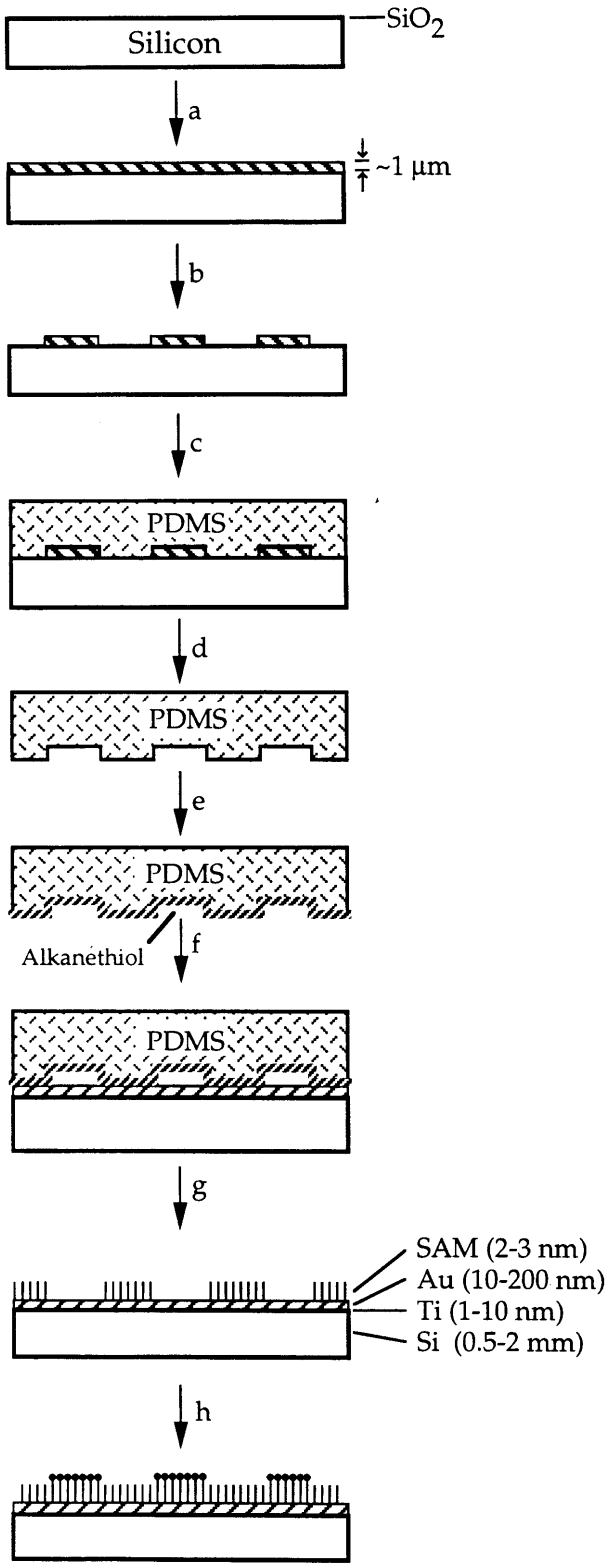


Figure 3. A schematic illustration of μCP for patterning monolayers on gold. Each step of the process is discussed in the text.

photoresist (c). The PDMS stamp (d) is inked with a solution of the alkanethiol in ethanol (e), dried and manually brought into contact with a gold surface (f); the alkanethiol is transferred only at those regions where the stamp contacts the surface (g). Conformal contact between the elastomeric stamp and surface and the rapid reaction of alkanethiols with gold permit the surface to be patterned over areas several square centimetres in size with edge resolution of the features better than 100 nm. The regions of gold that remain after the printing step can then be derivatized with a different SAM by immersing the substrate in a solution of a second alkanethiol (h). The least convenient step involves the photolithographic fabrication of the master pattern from which stamps are cast. Once prepared, multiple stamps can be cast from a single master and each stamp can be used hundreds of times. μCP has also been used to pattern alkylsiloxanes on the surfaces of SiO_2 and glass, but this method is not yet developed to the point of practical utility [33].

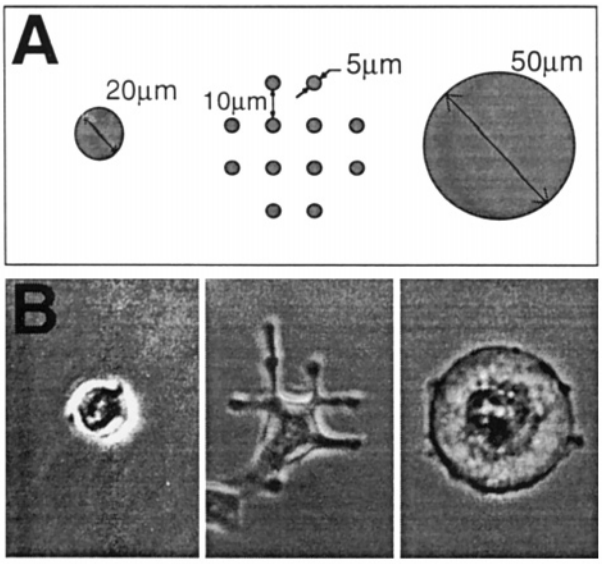


Figure 4. Adhesion of endothelial cells on monolayers patterned into regions terminated in methyl and hexa(ethylene glycol) groups. (*A*) Diagram of the patterns of monolayer used to control the shape and the total area over which cells contact matrix. (*B*) For sizes of adhesive circles with diameters greater than 20 μm, cells attached to a single patch and spread to the size of the patch; for smaller sized circles, cells attached to and spread on several patches [36].

Studies of cell growth on patterned substrates

The combination of μCP and SAMs of alkanethiolates on gold provides a convenient and flexible methodology for controlling the positions and shapes of cells attached to substrates [34]. The method uses μCP to print a pattern of hydrophobic SAM followed by immersion of the substrate in a solution of oligo(ethylene glycol)-terminated alkanethiol to render the nonprinted regions inert to protein adsorption and cell attachment. To insure efficient attachment of cells, these patterned substrates are immersed in a solution of matrix protein. Protein only adsorbs to the methyl-terminated regions: the oligo(ethylene glycol)-terminated regions resist entirely the attachment of cells [34]. This methodology can create substrates that control the shape – and hence the growth – of individual cells [35]. We have recently used this methodology to design substrates to determine whether apoptosis of adherent endothelial cells is prevented by the total area of contact between the cell and substrate or by the shape of the spread cell [36]. Figure 5 shows an example of a substrate patterned into several circles only a few microns in diameter: because the endothelial cells attached to several of these adhesive patches (but could not form adhesions to areas between the patches), the projected area of the cell differed from the area of adhesion. A systematic study of adhesion on several substrates showed that cell shape – and not the total area of underlying matrix – was the important determinant of apoptosis [36]. This methodology has also been used to control the attachment of endothelial cells to surfaces contoured into grooves and ridges [37]. I believe this methodology is currently the most flexible for controlling the interactions of cells with substrates and that it will become increasingly important as a tool in studies of cell-matrix interactions.

The use of patterned alkylsiloxanes on glass substrates has provided another important method for controlling cell adhesion [31, 38, 39]. Rudolph and co-workers, for example, observed that endothelial cells allowed to grow on lines having a width of 100 μm differentiated into neovascular cords after a period of several days; cells that were seeded on lines having widths of 200 or 500 μm, by contrast, had a less organized cytoskeletal structure [38].

Monolayers that present ligands

SAMs can be tailored with ligands, peptides and proteins. The design of substrates that present ligands for the biospecific recognition of receptors must at the same time prevent the nonspecific binding of other proteins. If proteins do adsorb to the substrate, the adsorbed protein can both mask the immobilized ligand and present other ligands from its primary sequence.

We have demonstrated that monolayers presenting oligo(ethylene glycol) groups and ligands are very effective for the biospecific adsorption of protein. As a first

system we investigated the binding of carbonic anhydrase to SAMs presenting the benzenesulphonamide group and tri(ethylene glycol) groups [18]. Our work used SPR as the analytical technique to measure adsorption. The ability of this technique to measure both kinetic and thermodynamic parameters for the association of soluble receptors with immobilized ligands, together with a commercial source for the instrument, makes it very well suited for these studies. SPR showed that carbonic anhydrase bound reversibly to these monolayers and that the amount of protein that bound was proportional to the density of benzenesulphonamide ligand in the SAM. Further, the adsorption was shown to be biospecific, since the addition of a soluble, competitive ligand to the buffer inhibited binding. This monolayer resisted the nonspecific adsorption of protein when presented with a solution containing nine different proteins at a total concentration of 2 mg/ml. Whitesides and coworkers have used a similar approach to study the recognition of immobilized D-Ala-D-Ala dipeptides by the antibiotic vancomycin [21]. This same strategy can be used to immobilize proteins to the monolayers. Sigal and co-workers used a monolayer that presented an Ni^{+2}-nitrilotriacetic acid complex and tri(ethylene glycol) groups to immobilize proteins whose primary sequence terminated in an oligo-histidine sequence [40]. This strategy has the advantages that the his-tagged proteins can be immobilized from impure samples (provided there are no other his-tagged proteins present), and the immobilized protein is presented in a uniform orientation. Hong and co-workers described a related strategy for presenting proteins in a uniform orientation. These authors used a monolayer terminated in the thiol group to immobilize a cytochrome c through a disulphide linkage [23]. The use of genetic engineering methods to generate mutants of the protein having a single cysteine residue on the surface allows the protein to be presented in a discrete orientation. The ease with which specific functional groups can be introduced into SAMs makes these interfaces compatible with nearly all immobilization chemistries (for a review of immobilization strategies, see ref. 41).

Functionalized substrates for cell adhesion

The finding by Ruoslahti and Pierschbacher that a key interaction in the adhesion cells on the fibronectin matrix involved binding of cellular integrin receptors to Arg-Gly-Asp (RGD) peptides made possible a class of simple substrates for cell adhesion [42]. Early work used polymers that were derivatized with RGD – or longer peptides that contain this sequence – to promote the adhesion of fibroblasts [43, 44]. In one example, polymer hydrogels modified with the GRGDS peptide supported the morphologically complete spreading of foreskin fibroblasts [43]. These examples provide a model system for understanding the relationships between the density and structure of immobilized ligands with the adhesion and spreading of cells. The use of polymer substrates, however, has the limitation that the environment of immobilized peptide is heterogeneous. Because not all of the peptides are accessible to cellular receptors – they may be buried in the polymer – it is difficult to control the density and homogeneity in binding strength of immobilized ligands. The regular structure of organic monolayers, by contrast, makes these substrates an excellent choice for mechanistic studies of cell adhesion.

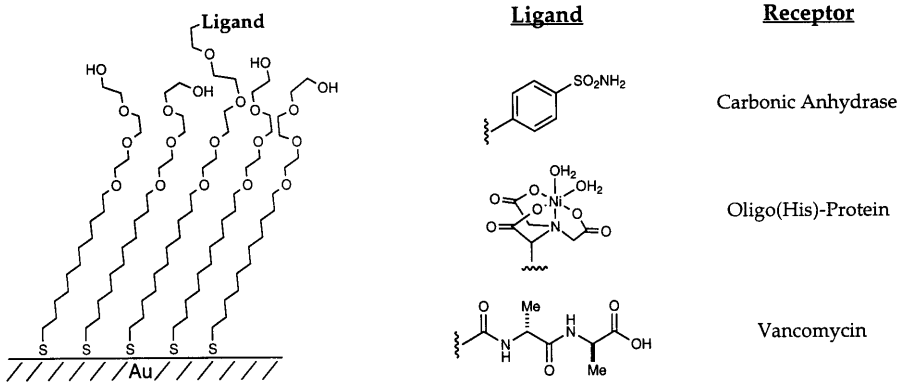


Figure 5. Monolayers for the biospecific adsorption of protein. (Left) General structure of a monolayer presenting a ligand and tri(ethylene glycol) groups. (Right) The ligand-receptor combinations that have been demonstrated with this system include binding of carbonic anhydrase to benzenesulphonamide; his-tagged proteins to a complex of Ni^{+2}; vancomycin to D-Ala-D-Ala.

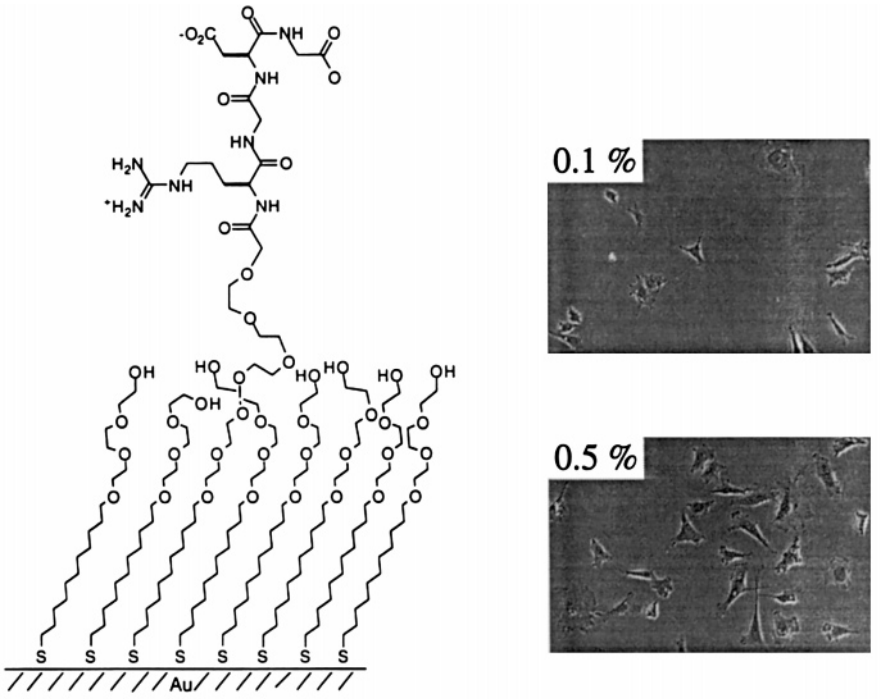


Figure 6. (Left) Structure of a monolayer presenting the Arg-Gly-Asp peptide ligand and tri(ethylene glycol) groups. (Right) Optical micrographs of 3T3 fibroblasts attached to monolayers presenting peptide ligand at densities of 0.1 and 0.5% (after a period of 5 hours).

Massia and Hubbell prepared alkylsiloxane monolayers that presented the RGD ligand in a homogeneous environment as substrates for the adhesion of human foreskin fibroblasts [45]. These authors found a strong dependence of adhesion on the density of peptide. At surface densities of peptide equal to 1 fmol/cm^2, the fibroblasts attached to and spread on the substrates, but did not form stress fibres and focal adhesions. At a 10-fold higher density of peptide, cells did recruit these structures. It was difficult to correlate the behaviour of these cells over longer periods of time because of the possibility that the cells could remodel the matrix – that is, that they could replace the underlying matrix proteins with secreted matrix proteins.

We have used SAMs of alkanethiolates on gold that present the GRGD peptide ligands and oligo(ethylene glycol) groups as substrates with which to study cell adhesion (fig. 6) [46]. For a series of monolayers having densities of RGD peptide decreasing from 1 to 0.001%, the number of attached endothelial cells decreased and the degree to which the cells spread varied. Fluorescence immunostaining showed that attached fibroblasts formed focal adhesions and stress fibres (fig. 7) (B. T. Houseman and M. Mrksich, unpublished observations). Two experiments suggest that while these monolayers allow cells to attach, they resist the deposition of matrix proteins by the cells. SPR showed that monolayers presenting the peptide ligand at a density of 0.5% resisted the adsorption of several proteins [46]. Further, when cells were cultured in the presence of radiolabelled amino acids, the amount of matrix deposited on the substrate was substantially reduced compared with that on conventional substrates that had adsorbed fibronectin.

Dynamic substrates

This review has described an important strategy for creating substrates that have well-defined structures. The extension of this methodology to create substrates whose structures and properties are under dynamic control – for example, the ability to release adsorbed proteins or to change the presentation of ligands – will provide an important method for further studies. A number of early examples have been described. Although these methods are not yet developed to the point of practical utility, they do provide a preview of the types of tailored substrates that will be developed over the next several years.

Okano and co-workers used poly(*N*-isopropylacrylamide) gels grafted to polystyrene dishes as substrates for the adhesion of endothelial cells and hepatocytes [47]. These gels undergo a phase transition at tempera-

tures below 20 °C to give a material that resists the adsorption of protein. Consequently, when the culture dishes were removed from an incubator, the adherent cells were released from the dishes in a period of 30 min. We have developed a strategy that can selectively release individual ligands from a monolayer of alkanethiolates on gold [48]. The method attaches the ligand to the monolayer through a redox-active group that undergoes electrochemical oxidation (using the gold as working electrode) and subsequent cleavage to release the attached ligand. Because the monolayer is stable to the applied electrical potentials, it will be possible to create substrates that present multiple groups but selectively release only a fraction of these groups.

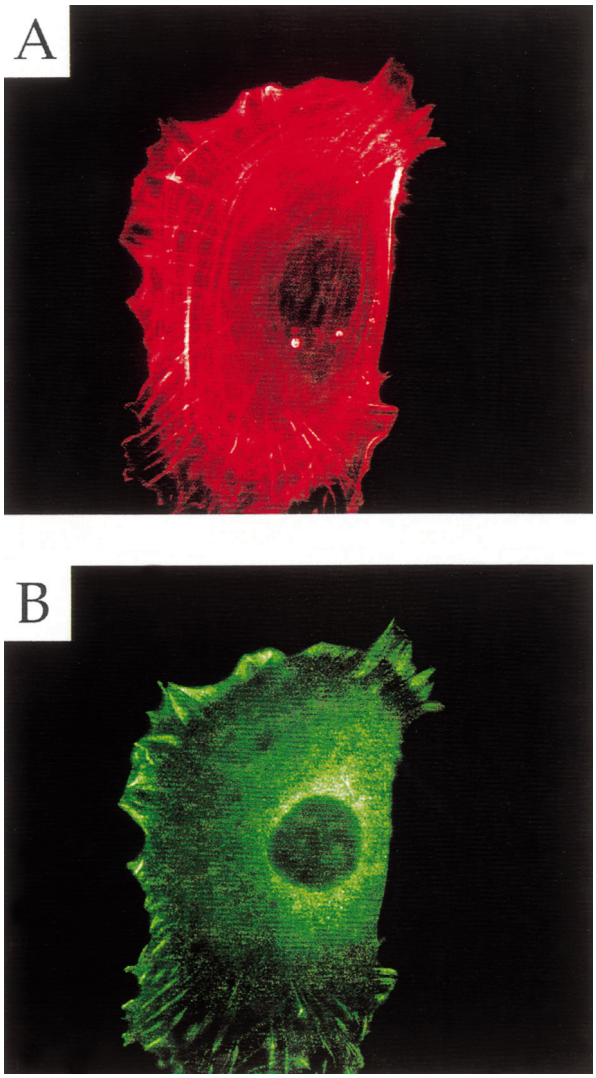


Figure 7. Fluorescence immunostaining of F-actin (*A*) and vinculin (*B*) in a fibroblast attached to a monolayer presenting 1% RGD peptide as illustrated in figure 6. Cells were fixed for 30 min in 4% paraformaldehyde/phosphate-buffered saline and permeabilized with 0.1% Triton X-100. Labelling was performed using rhodamine-conjugated phalloidin and a fluorescein immunoglobulin G conjugate to monoclonal antivinculin.

Electrical fields present at the interface of a conducting substrate can affect the behaviour of cells. Langer and co-workers used electrically conducting polypyrrole films as substrates for studies of neurite outgrowth in PC-12 cells [49]. Cultured cells subjected to an electrical stimulus produced neurite lengths that were greater by a factor of 2 relative to those cultured in the absence of fields. A related study showed that reversible oxidation of these polymer films could stall the growth (including cell extension and DNA synthesis) of aortic endothelial cells [50]. Dynamic fields generated by applying an AC current to regions of a substrate have been shown to repel cells in suspension and prevent adhesion [51]. The mechanisms by which applied fields can influence the behaviour of cells are not yet well understood, but the ability to control both the spatial localization and intensity of fields offers many opportunities for creating functional substrates.

Techniques common to silicon microfabrication provide access to a range of tailored substrates that can measure mechanical and physiological properties of cells [52]. Galbraith and Sheetz, for example, used micromachining to create a substrate that had several thousand micron-sized pads that could measure forces exerted by an attached cell [53]. They used this substrate to measure the distribution of traction forces exerted on a substrate by a migrating fibroblast [53]. In a programme to develop cell-based sensors, Kovacs has fabricated electrode arrays for measuring the response of attached neural cells to chemical toxins [54].

SAMs of alkanethiolates on gold are currently the best class of substrates that can control the structure, density and pattern of immobilized ligands. The ease with which these substrates can be prepared, the synthetic flexibility available in attaching different groups, and the compatibility with the conditions and techniques of cell culture make this methodology broadly useful for studies of attached cell culture. This review highlighted several examples that utilized tailored substrates for studies in experimental cell biology. The continued collaborations of biologists, chemists and surface scientists will indeed lead to many more examples.

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