Using Microcontact Printing to Pattern the Attachment of Mammalian Cells to Self-Assembled Monolayers of Alkanethiolates on Transparent Films of Gold and Silver

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This paper describes a convenient and general methodology to pattern the attachment of anchorage-dependent cells to glass coverslips modified with an optically transparent film of gold or silver and a self-assembled monolayer (SAM) of alkanethiolates [1–3]. This work uses microcontact printing (μCP) [4, 5] to pattern SAMs into regions terminated in methyl groups and tri(ethylene glycol) groups; SAMs terminated in methyl groups promote the hydrophobic adsorption of protein and SAMs terminated in oligo(ethylene glycol) groups resist essentially entirely the adsorption of protein. Immersion of these patterned substrates in a solution containing fibronectin results in adsorption of protein specifically on the methyl-terminated regions of the SAM; subsequent placement of these substrates in medium containing a suspension of bovine capillary endothelial (BCE) cells results in the attachment and spreading of cells predominantly on the regions of the patterned SAM that present fibronectin.

Cell adhesion is important for control of cell shape, growth, and function. Although it is not yet routine to experimentally vary the adhesion of cells in a controlled manner, several groups have developed methods to control spatially the attachment of cells to substrates [6–20]. Much of this work has employed glass slides that were modified with monolayers of alkylsiloxanes and has used photolithographic methods to pattern these monolayers. For example, Kleinfeld and coworkers patterned quartz substrates into alkylsiloxanes terminated in methyl groups and aminogroups with sizes of features down to 5 μm [9]. When plated in the presence of serum, cerebellar cells attached only to the amino-terminated regions of the substrate; we presume that proteins of the serum that do not support cell attachment adsorbed to the methyl-terminated regions. Though this and much other work has provided a general method to control the attachment of cells to surfaces, the methodology is limited in three ways: (i) by the technical requirements of photolithography—the equipment and controlled environment facilities are not routinely available to cell biologists; (ii) by the control over the interfacial properties of the alkylsiloxanes—it is not straightforward to synthesize alkylsiloxanes with many common organic functional groups.

INTRODUCTION

This paper describes a convenient and general methodology to pattern the attachment of anchorage-dependent cells to glass coverslips modified with an optically transparent film of gold or silver and a self-assembled monolayer (SAM) of alkanethiolates [1–3]. This work uses microcontact printing (μCP) [4, 5] to pattern SAMs into regions terminated in methyl groups and tri(ethylene glycol) groups; SAMs terminated in methyl groups promote the hydrophobic adsorption of protein and SAMs terminated in oligo(ethylene glycol) groups resist essentially entirely the adsorption of protein. Immersion of these patterned substrates in a solution containing fibronectin results in adsorption of protein specifically on the methyl-terminated regions of the SAM; subsequent placement of these substrates in medium containing a suspension of bovine capillary endothelial (BCE) cells results in the attachment and spreading of cells predominantly on the regions of the patterned SAM that present fibronectin.

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and there still exists no class of alkylsiloxanes that resists efficiently the adsorption of protein; (iii) by the difficulty in using photolithography to attach to the surface the complicated or delicate ligands required for experiments based on biospecific adsorption. New methods to generate reactive functional groups on the surface to immobilize proteins will alleviate some of these problems [21–23].

We have described previously the use of patterned monolayers of terminally substituted alkanethiols on the surface of gold to control the attachment and spreading of rat hepatocyte and rat basophilic leukemia cells [14, 17]. Our previous reports did not address several important aspects of the system, including the efficiency of matrix adsorption, the long-term stability of the patterned SAMs in culture, optimal methods for handling the cells and substrates, and the development of transparent substrates that could be used in conjunction with light and fluorescence microscopy. In this report we address these issues directly and we discuss the prospects of this methodology for cell culture and cell biological studies.

Self-assembled monolayers on gold and silver. SAMs of alkanethiols form upon the adsorption of long-chain alkanethiols from solution onto the surfaces of gold and silver [1–3]. SAMs on gold are the best characterized [24]. The sulfur atoms are proposed to attach to the hollow, threefold sites of the gold (111) surface [25, 26], although a recent report suggested that the sulfur atoms attach as disulfides [27]; in either case, the alkyl chains are predominantly trans-extended, close-packed, and tilted approximately 30° from the normal to the surface (Fig. 1). Characterization of the crystallographic texture of evaporated films of gold suggest that greater than 80% of the film exhibits the (111) texture [28]. The oriented alkyl chains present the terminal functional group at the interface; the properties of the interface are largely determined by this group. Even complex and delicate groups can be introduced through straightforward synthesis [for examples, see 29 and 30]. SAMs on silver differ primarily from those on gold in that the alkyl chains are oriented nearly perpendicular to the surface [31]. SAMs can be prepared on optically transparent films of gold or silver having a thickness of 10 nm [32]; these substrates are especially useful for biological applications, where inverted optical microscopy is required.

Adsorption of protein on SAMs. The adsorption of protein on hydrophobic SAMs of hexadecanethiolate (—S(CH₂)₁₅CH₃) is usually rapid and irreversible [33–35]. We have found that SAMs presenting oligomers of the ethylene glycol group [(—CH₂CH₂O—)ₙR, n = 3 – 7, R = H or CH₃] are very effective at resisting the adsorption of protein [33–35]. SAMs presenting even the short tri(ethylene glycol) group successfully resist the adsorption of “sticky” proteins such as fibrinogen.

Patterned SAMs using microcontact printing. We have developed a convenient technique—microcontact printing—that can pattern the formation of SAMs in two dimensions [4, 5]. This technique uses an elastomeric stamp to transfer an alkanethiol to designated regions of a surface of gold or silver (Fig. 2). The stamps are usually fabricated by pouring a prepolymer of polydimethylsiloxane (PDMS) onto a master pattern containing the pattern in relief; this master is often formed by photolithographic methods, but other sources are available—e.g., diffraction gratings, micromachined structures, and replicas of other microstructures. The stamp is inked with a solution of the alkanethiol, the solvent is allowed to evaporate, and the stamp is brought into contact with the surface; the SAM forms only at those regions where the stamp contacts the surface. A SAM presenting a different group can be formed at the complementary regions byimmersing the substrate (after removal of the stamp) in a solution of a different alkanethiol. Microcontact printing can form patterns with dimensions of features down to 1 μm conveniently and down to 200 nm in special cases. Because μCP relies on self-assembly and does not require a controlled laboratory environment, it is both more convenient and more economical as a method for producing patterned substrates than is photolithography. It is also applicable to the formation of SAMs presenting structurally complex terminal groups. Its principle disadvantages are that it requires the thin gold films as starting materials and that the durability of the SAMs of alkanethiols on gold or silver may be less than alkylsiloxanes on glass or silica.

MATERIALS AND METHODS

Preparation of SAMs and patterning using μCP. Substrates were prepared by evaporation (using an electron beam to heat the sources) of thin films of titanium (1.5 nm) and gold (12 nm) or silver (12 nm) on glass coverslips (0.20 mm thick, No. 2, Corning). Nonpatterned substrates were formed by immersing these metalized slides in solutions of alkanethiol (either hexadecanethiol (HS(CH₂)₁₅CH₃) or HS(CH₄)₁₁(OCH₂CH₂)₃OH) in ethanol (2 mM)
SAMs of alkanethiolates

Coating substrates with fibronectin. Patterned coverslips were placed in sterile petri dishes containing phosphate-buffered saline (PBS: 20 mL, pH 7.4) after which a solution of fibronectin (FN; 111 µg/mL) (Organon Teknika Cappel, Malvern, PA) was added and gently dispersed to give a final concentration of 25 µg/mL. After an incubation of 2 h at room temperature, the solution of fibronectin was diluted by adding ~140 mL of PBS. The slides were removed from the solution under a stream of PBS and transferred immediately (without letting the slides dry) to petri dishes containing 1% bovine serum albumin (BSA) in Dulbecco's modified Eagle's medium (DMEM) (10 mL).

Cell culture. Bovine capillary endothelial cells were isolated from adrenal cortex and cultured as described previously [40, 41]. Cell monolayers were dissociated by brief exposure to trypsin-EDTA, washed with 1% BSA/DMEM, and resuspended in 2× defined medium. One-half volume of 1% BSA/DMEM was removed from the petri dishes containing coverslips and replaced by an equal volume of 2× defined medium containing cells (7.5 × 10^5 cells/mL) (final concentration of components in defined medium: 10 µg/mL high-density lipoprotein (HDL), 5 µg/mL transferrin, 5 ng/mL fibroblast growth factor (FGF), 1% BSA). After 2 h of plating, 75% of the medium was removed and replaced with fresh defined medium. The medium was exchanged at 24 and 96 h after plating. In designated experiments, after 24 h of plating, defined medium was replaced with normal culturing medium containing 10% calf serum (CS) and FGF (0.5 ng/mL). Cultures were maintained over a 5- to 7-day period and photographed using a Nikon Diaphot phase-contrast microscope (100× magnification) and T-max film.

Immunofluorescence microscopy. Cells were cultured for extended periods of time (24, 48, and 72 h postplating) as described. Actin microfilaments were identified by fixing cells with 4% paraformaldehyde for 30 min at room temperature and staining with fluorescein-labeled phalloidin (Sigma) diluted 1:1000 with IF buffer (0.2% Triton X-100, 0.1% BSA, PBS). Immunofluorescent images were photographed using a Zeiss Axiomat microscope and Kodak T-max film (630× magnification).

Quantitation of adsorbed fibronectin by ellipsometry. Substrates were prepared by evaporating titanium (1.5 nm) and gold (100 nm) on silicon wafers (Silicon Sense, Nashua, NH). The wafer was cut into pieces about 1 cm^2 in size and these samples were immersed in a solution of hexadecanethiol in ethanol (2 mM) for 12 h. The slides were removed from solution, rinsed with ethanol, and dried with nitrogen; the ellipsometric constants were recorded and the slides were placed in scintillation vials containing PBS buffer (1.8 mL). A 10× solution of fibronectin in PBS (200 µL, final concentrations of 0.5, 5, 25, 50, and 500 µg/mL) was added to the SAMs in PBS. Each set of five substrates was left in solution for 0.5, 1, 2, 4, or 24 h. The solutions were then diluted by adding 10 vol of buffer, and the substrates were removed under a flow of buffer, rinsed with water, and dried with nitrogen. The ellipsometric constants were recorded and compared with the initial values to give an average value of thickness of the adsorbed protein. Ellipsometric measurements were made with a Rudolf Research Type 43603-200 E manual thin-film ellipsometer operating at 632.8 nm (He–Ne laser) and an angle of incidence of 70°. Values for the average thickness of the protein layer were calculated using a planar, three-layer (ambient–protein–SAM) isotropic model with assumed refractive indices of 1.00 and 1.45 for the ambient and the protein, respectively, as described previously [34].

RESULTS

Quantitation of adsorption of fibronectin. To determine experimental conditions that effectively coated
FIG. 3. Relationship between the adsorption of fibronectin on SAMs of hexadecanethiolate and the concentration of protein and time of adsorption. Increasing concentrations of fibronectin were allowed to adsorb to SAMs of hexadecanethiolate and the average thickness of adsorbed protein was determined using ellipsometry. Data are shown for several concentrations of protein—0.5 μg/mL (●); 5 μg/mL (○); 25 μg/mL (■); 50 μg/mL (□); 500 μg/mL (▲)—and different periods of time—0.5, 1, 2, 4, and 24 h.

SAMs of hexadecanethiolate with fibronectin, we measured the amount of protein that adsorbed using different concentrations of protein (from 0.5 to 500 μg/mL) and allowed the protein to adsorb for different periods of time (from 0.5 to 24 h) from PBS buffer. We used ellipsometry to measure the average thickness of the protein layer adsorbed on the SAM; this technique relates changes in the polarization of ellipsometrically polarized light that is reflected from the interface to the amount of protein adsorbed at the interface. Ellipsometry is a rapid and sensitive technique, and it does not require the protein to be radiolabeled [42]. Figure 3 shows that allowing the substrates to adsorb fibronectin for 2 h from a solution having a concentration of the protein of 25 μg/mL is sufficient to provide a dense layer of fibronectin with minimal waste of protein.

Adsorption of fibronectin to patterned SAMs. We used μCP to pattern substrates with a set of parallel lines of methyl-terminated SAM and separated by regions of tri(ethylene glycol)-terminated SAM 120 μm in width. We employed patterns having four different widths of lines of the methyl-terminated SAM: 10, 30, 60, and 90 μm. The patterned substrates were placed in petri dishes containing PBS and a 10× solution of fibronectin was added and allowed to stand for 2 h. In order to confine the protein to the pattern of SAM, we found that it was necessary to dilute the protein-containing solution with excess buffer (~7–10 vol) and then to remove the slides from the solution under a stream of buffer delivered from a pipette. Figure 4 shows an image obtained by scanning electron microscopy of the pattern of fibronectin on the substrate [43]. The pattern of protein conforms well to the pattern of SAM, with an edge resolution better than 1 μm.

Attachment of cells on patterned SAMs. After substrates had been coated with fibronectin, they were placed in petri dishes containing defined medium (10 mL). Endothelial cells were added to the slides in defined medium, allowed to adhere for 2 h, exchanged with medium containing 10% calf serum, and maintained in culture for several days. After 48 h, cells were observed to attach to the patterned substrates only at regions that presented fibronectin. Figure 5 shows optical micrographs of cells attached to each of the four patterns having adhesive lines with dimensions of 10, 30, 60, and 90 μm. The images show cells attached preferentially to nonpatterned regions and to regions patterned into lines. In all cases, cells attached to the regions of SAM presenting methyl groups and immobilized fibronectin; the regions of SAM presenting tri(ethylene glycol) groups resisted the attachment of cells.

Growth of cells attached to patterned SAMs. Figure 6 shows a time course of the cells attached to a patterned SAM having lines 60 μm in width. After 6 h, the density of attached cells was low but the pattern was already evident. The cells grew to near confluence after a period of 24 h and reached a confluent monolayer at 72 h. After a period of 5 days, the cells were still viable, but were beginning to invade areas of the substrate presenting tri(ethylene glycol) groups. We believe that this behavior is due to an extension of the confluent layer—perhaps by the cells extending the insoluble matrix—rather than attachment of cells to

FIG. 4. Fibronectin (100 μg/mL in PBS) was allowed to adsorb to a patterned SAM for 2 h. The pattern of protein was characterized by scanning electron microscopy. The layer of protein attenuates secondary electron emission and appears as the dark areas in the micrograph. The inset shows a view at higher magnification and demonstrates the edge resolution of the pattern of protein.
proteins adsorbed at the surface, because cells invaded only the inert regions where the borders of adhesive regions met at a right angle and not along straight borders. Fluorescence immunostaining of the actin microfilaments (discussed below) also supports this interpretation.

**Attachment of cells to patterned SAMs on silver.** Figure 7 shows cells attached to patterned SAMs supported on a thin film of silver after a period of 24 h. The pattern of cells in this image conforms well to the pattern of SAM. While the patterning of cells on these substrates was often nearly identical to that on the gold substrates, we did find over the course of many experiments that the patterning of cells was less effi-

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**FIG. 5.** Optical micrographs of cells attached to patterned SAMs on gold having unpatterned regions terminated in methyl groups (left) and patterned lines (right) with sizes of 10 (A), 30 (B), 60 (C), and 90 (D) μm. Regions of the surfaces that are modified with SAMs presenting tri(ethylene glycol) groups resist the adsorption of protein and subsequent attachment of cells. We presume that the uneven patterning in (B) was due to a defect in the PDMS stamp used to pattern the SAM and not by loss of fidelity in the procedure for μCP. Cells were plated and maintained in medium containing 10% calf serum. These photographs were taken after 48 h in culture. The scale bar applies to all images.

**FIG. 6.** Optical micrographs of cells attached to patterned SAMs on gold having lines 30 μm in width. These images were taken after 6 h (A), 24 h (B), 48 h (C), and 5 days (D) in culture. The scale bar applies to all images.
FIG. 7. Optical micrograph of cells attached to patterned SAMs of alkanethiolates on silver having lines with sizes of 10 μm (A) and 30 μm (B) and separated by inert regions 120 μm in width. The cells were plated and maintained in defined medium. The scale bar applies to both images.

cient than observed with substrates supported on thin films of gold (data not shown); in many regions the cells did not attach to the substrate, or the cells invaded the "inert" regions of the substrate. There are several possible explanations for the lower fidelity observed with the SAMs on silver: SAMs of alkanethiolates on silver may be less stable than are those on gold under conditions of cell culture; inorganic silver salts may be released from the substrate and are toxic to cells; and SAMs of alkanethiolates presenting oligo(ethylene glycol) groups on silver may be less effective at preventing the adsorption of protein than are those on gold.

Immunofluorescent staining for actin of attached cells. We determined the suitability of these substrates in assays that use optical fluorescence microscopy. Cells were maintained in medium containing 10% calf serum, fixed after 24 and 72 h in culture, and stained with fluorescein-labeled phalloidin. Phalloidin recognizes F-actin and is used to label actin microfilaments of the cytoskeleton. Figure 8 shows optical micrographs of the actin microfilaments of cells adherent to patterned lines 30 μm in width. The microfilaments after 24 h of culture displayed an organization typical of nonpatterned cells, although the filaments were aligned at the edges of the patterns. After 72 h in culture the microfilaments were uniformly aligned with the underlying pattern (Fig. 8). Figure 9 shows an optical micrograph of the actin microfilaments of cells adherent to intersecting lines of the pattern. The microfilaments of adherent cells reoriented to align with the intersecting, perpendicular patterned line. The micrograph also shows a filament that bridges the inert region of the substrate. This filament, however, is not in the same focal plane as the others because it does not make adhesive contacts to the substrate [40].

DISCUSSION

μCP permits control over the formation of patterns. The use of μCP for patterning the attachment of cells to surfaces provides several advantages over photolithographic methods that have been employed previously. Microcontact printing is an inexpensive procedure; it does not require the equipment and controlled environments that are common for photolithographic patterning. It is experimentally simple; a single substrate can be patterned by μCP in minutes. The variety of patterns that can be produced by μCP is limited only by the availability of master patterns from which the elastomeric stamps are cast; stamps having feature
sizes down to 1 μm are easily generated. Obtaining the master patterns—these are often produced using photolithography—is the least convenient step in the procedure, although hundreds of stamps can be cast from a single master, and each stamp can be used hundreds of times. Even nonplanar and curved substrates can be patterned using μCP [44]. Microcontact printing has recently been extended to patterning the formation of alkylsiloxanes on hydroxylated surfaces (glass and silicon oxide) [45]; we believe that these procedures will be developed soon to the point of utility for biological studies.

Fabrication of patterned SAMs on gold and silver. The primary limitation with this methodology is obtaining the thin films of gold or silver on glass slides. The electron-beam evaporation systems are still expensive and not routinely available. The masters—typically a silicon wafer having a pattern of photoresist—from which PDMS stamps are fabricated are often prepared by photolithography using a mask imprinted with the pattern to be reproduced. These photolithographic masks are routinely available through commercial suppliers, and commercial photolithography laboratories can produce the master substrates, though these services are not yet inexpensive.

Stability of patterned SAMs in cell culture. SAMs of alkanethiolates on gold were stable in cell culture for periods of 5–7 days and the pattern of cells was persistent over this period of time. The gold and organic thiols appear not to be toxic to the cells. SAMs do desorb, however, on heating to temperatures greater than 100°C [43] and on exposure to UV radiation in the presence of oxygen [47], and care must be taken to protect the substrates from intense light or excessive temperatures prior to and during experiments. Because of the limited stability under extreme temperatures, these substrates cannot be sterilized with an autoclave.

SAMs on transparent films of gold. The optical transparency of the substrates used in this work make them well suited for many investigations of cultured cells. The thin films of gold and silver do not obscure images of attached cells when observed by inverted optical microscopy. These substrates are also compatible with routine immunofluorescent staining; the thin film of gold does not significantly quench the fluorescence. Because SAMs can form on most surfaces of gold, this methodology is compatible with any substrate on which a thin film of gold can be prepared. We have employed substrates made of glass, silica, polyurethanes, and polystyrenes; we believe that many other materials will also be useful as substrates.

SAMs on silver. SAMs of alkanethiolates on silver were less effective than those on gold at controlling the attachment of cells over periods greater than 2–3 days; cells were observed to invade inert regions—that is, regions of SAM presenting tri(ethylene glycol) groups—and in several cases attached less efficiently to the protein-coated regions. Further studies are necessary to understand the reasons for these differences. The structures of SAMs on gold and silver differ predominantly in the orientation of the alkyl chains relative to the substrate; the alkyl chains are oriented
nearly perpendicular to silver supports [30], and ~30° from the normal on gold supports [24]. The greater density of ethylene glycol units on silver may make them less effective at resisting the adsorption of protein over longer periods of time.

**SAMs permit flexible control over the surface chemistry.** SAMs of alkanethiolates provide a well-defined and synthetically flexible system with which to control the properties of surfaces. In this work, SAMs presenting methyl groups were used to immobilize fibronectin and SAMs presenting oligo(ethylene glycol) groups were used to passivate the surface toward protein adsorption and subsequent cell attachment. SAMs terminated in methyl groups provide a hydrophobic surface to which proteins rapidly adsorb. For these SAMs to support the attachment of cells, they must present the appropriate matrix proteins. We therefore allowed fibronectin to adsorb to the SAM before introducing the substrate to a suspension of cells. We [14, 32] and others [9] have found that hydrophobic surfaces, when not treated in this way with matrix protein, do not support the attachment of cells. We presume that proteins in the medium that do not support adhesion adsorb preferentially to the substrate.

The oligo(ethylene glycol) group is currently the best available at controlling unwanted protein adsorption; it resists the adsorption of virtually all proteins under a range of conditions; it even resists the in situ adsorption of the "sticky" protein fibrinogen [33–35]. This group, however, is not unique in its ability to prevent adsorption; we have recently found that SAMs terminated in the tri(propylene sulfoxide) group also resist the adsorption of protein, though the mechanisms for resistance are not completely understood [48]. We note that there are other systems of functional groups and surfaces that form good monolayers: alkylphosphonates on zirconium oxide [49], alkylhydroxamic acids on silver oxide [50], and alkylsulfonitriles on platinum [51]. These systems may also be well suited as substrates for cell culture, but they have not yet been explored in this application.

**Implications.** The combination of SAMs and μCP described here provides a remarkably convenient system with which to control the attachment of cells to substrates for biological studies. The results from this work are appropriate for controlling the shapes and positions of attached cells to planar substrates. We believe that SAMs offer an even greater opportunity for tailoring the molecular composition of substrates with the sorts of ligands that are important for control over the activity of attached cells; these substrates will be useful both for fundamental investigations of mechanisms of signal transduction between a cell and ligands of the substrate and for controlling the metabolic activities of attached cells.

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