

Using Self-Assembled Monolayers to Pattern ECM Proteins and Cells on Substrates

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1. Introduction

We present a method that uses microcontact printing of alkanethiols on gold to generate patterned substrates presenting “islands” of extracellular matrix (ECM) surrounded by nonadhesive regions such that single cells attach and spread only on the adhesive regions. We have used this micropatterning technology to demonstrate that mammalian cells can be switched between growth and apoptosis programs in the presence of saturating concentrations of growth factors by either promoting or preventing cell spreading (1). From the perspective of fundamental cell biology, these results suggested that the local differentials in growth and viability that are critical for the formation of complex tissue patterns may be generated by local changes in cell–ECM interactions. In the context of cell culture technologies, such as bioreactors and cellular engineering applications, the regulation of cell function by cell shape indicates that the adhesive microenvironment around cells can be carefully optimized by patterning a substrate in addition to using soluble factors (2). Micropatterning technology will play a central role both in our understanding how ECM and cell shape regulate cell physiology and in facilitating the development of cellular biosensor and tissue engineering applications (3–5).

Historically, investigations of cellular responses to various adhesive environments were limited by a lack of control over the interfacial properties and the topology of available substrates. It was particularly difficult to generate substrates patterned with adjacent adhesive and nonadhesive regions. In the past decade, the technology to engineer the properties of a surface with molecular-level control and to pattern these substrates with ligands suitable for

biological experiments has advanced rapidly. This progress was obtained as a result of the modification of microfabrication techniques used in the electronics industry in conjunction with polymer science and surface science. This powerful class of “micropatterning” techniques makes it possible to pattern surfaces with defined reactivity and topography with varying degrees of precision, depending on the methods used.

Surfaces with spatially patterned chemical functionalities can be formed using several techniques: vapor deposition, photolithography, and microcontact printing. Vapor deposition of metals through a patterned grid onto polyhydroxyethyl methacrylate (pHEMA) produces a substrate that presents complementary patterns of metal and pHEMA (6,7). Cells attach selectively to the metallic regions because they adhere to the metal (or more properly, to proteins adsorbed on the metals), but not the pHEMA; however, the edge resolution of this method is low (5 μm). Photolithography, which uses ultraviolet light to illuminate photosensitive materials through a patterned mask, can routinely produce patterns of defined chemical properties with resolutions better than 1 μm . To generate surfaces with only selected regions that promote cell attachment, various investigators have lithographically photoablated proteins preadsorbed to a silicon or glass surface (4); uncovered protein-adsorbing regions of a substrate previously coated with photoresist (8); or covalently linked proteins onto photoactivatable chemicals on the surface (9). A major limitation of these approaches is that the “nonadhesive” regions of the pattern are usually surfaces that actually promote the adsorption of protein, and require passivation (blocking of adhesive sites) with a protein such as albumin that prevents the adsorption of ECM proteins and the adhesion of cells. Over a period of days, however, cells are able to migrate onto these regions, probably as a result of degradation of the albumin and deposition of ECM by cells. Several investigators have tried to avoid these problems by using photolithography to pattern monolayers of trichloroalkylsilanes chemisorbed on the surface of SiO_2 . Self-assembled monolayers (SAMs) of alkylsiloxanes which present regions of perfluoro- and amino-terminated moieties promote cells to attach in patterns onto the surface; the amino-terminated siloxane promotes the preferential adhesion of cells and the perfluoro-terminated regions resist adhesion without passivation with albumin (5,10–12). Several technical issues remain in using this approach. This type of SAM is not easy to form, and a variety of biologically relevant organic functional groups (e.g., peptides and carbohydrates) are not compatible with the conditions used for its formation, thus limiting the range of surface chemistries available. The mechanism by which cells adhere to SAMs of alkylsiloxanes terminated with amine groups has not been elucidated, although, again, adsorption of proteins from the culture medium onto the charged surface is a plausible first step. Despite these short-

comings, this approach is still a viable one to be used for patterning the adhesion of cells.

Recent advances in the study of SAMs of alkanethiolates on gold surfaces has provided a more versatile approach to the patterning of cells. These SAMs are highly ordered molecular assemblies that chemisorb on the surface of gold with nearly crystalline packing to produce a new interface whose properties are determined solely by those of the head-group of the alkanethiol (*13*). This system makes it possible to control the interfacial properties of surfaces exposed to cells with greater molecular-level detail than other methods, and it affords the chance to influence cellular adhesion with greater specificity than with other methods (*14,15*). The synthetic procedures used to make alkanethiols are compatible with complex ligands that interact biospecifically on the cell (*15,16*). Alkanethiols can be patterned easily on a gold surface using microcontact printing (μ CP), a technique in which a flexible polymeric stamp is used to print the alkanethiols in a specified pattern; the size of the stamped regions can be defined arbitrarily with dimensions from 500 nm (or, with greater experimental difficulty from 200 nm) and up (*17*). After printing a hydrophobic alkanethiol, the remaining bare surface of the gold is exposed to an alkanethiol that presents tri(ethylene glycol) groups (e.g., $\text{HS}(\text{CH}_2)_{11}\text{O}(\text{CH}_2\text{CH}_2\text{O})_2\text{CH}_2\text{CH}_2\text{OH}$) that resist the adsorption of proteins. Thus, a pattern of these two SAMs presented on a substrate defines the pattern of ECM that adsorbs from solution onto the substrate (*3,18*). The hydrophobic SAMs created on flat gold substrates pattern the otherwise nonspecific adsorption of ECM proteins (fibronectin, fibrinogen, vitronectin, collagen I, and laminin) that promote the adhesion of different cell types (bovine capillary endothelial and rat hepatocytes) to the surfaces, whereas the tri(ethylene glycol) SAMs resist protein adsorption and cell adhesion (*1,3,18–22*).

Here, we describe how to use μ CP to fabricate substrates that present patterned SAMs with features >500 nm; features as small as 200 nm can be obtained in special cases, but they are not necessary for most conventional biological applications (*23*). This technique uses an elastomeric stamp with bas-relief to transfer an alkanethiol to the surface of gold in the same pattern defined by the stamp. The stamps are usually fabricated by pouring a prepolymer of polydimethylsiloxane (PDMS) onto a master relief pattern, which is often formed by photolithographic methods. Because μ CP relies on self-assembly of an alkanethiol, it does not require a dust-controlled laboratory environment, and can produce patterned gold substrates at relatively low cost.

2. Materials for Microcontact Printing

2.1. Glass Substrates Coated with Titanium then Gold

Microscope slides (Fisher, Pittsburgh, PA, no. 2) are loaded on a rotating carousel in an electron-beam evaporator (most of these are partially home

built). Evaporation is performed at pressures $<1 \times 10^{-6}$ torr. Occasionally, during the evaporation of titanium, the pressure increases above 1×10^{-6} torr, but decreases after allowing the chamber to stabilize for approximately 2 min. Allow the metals to reach evaporation rates of 1 \AA/s . Allow 400–500 \AA of each metal to evaporate before opening the shutters and exposing the glass slides to 15 \AA of titanium (Aldrich, Milwaukee, WI, 99.99+% purity) and 115 \AA of gold (Materials Research Corporation, 99.99 + % purity).

2.2. PDMS Stamp with Patterns Molded from a Photolithographic Master

Basic lithographic techniques, concepts, and terminology are described by Madou (24). Procedures that result in thicker features are available from the manufacturers of other types of photoresists; for the sake of brevity, we do not describe them here. Use test grade N type, 9–13-mils thick silicon wafers (Silicon Sense, Nashua, NH), with $\langle 100 \rangle$ orientation, phosphorus dopant, and 1–10 resistivity.

2.2.1. Generating Silicon Master with Desired Pattern Using Photolithography

In a clean room (preferably Class A), clean the wafers by sonicating for 5 min successively in trichloroethylene, acetone, then methanol. Bake at 180°C for 10 min to dry thoroughly. Spin coat (40 s @ 4000 rpm) the wafers with approx 1–2 mL hexamethyldisilazane (Shipley) followed by Shipley 1813 positive photoresist (40 s @ 4000 rpm produces a layer of 1.3 μm); bake the resist at 105°C for 3.5 min. Expose the wafer on a mask aligner (typically a Karl Suss model) through a photomask with features etched in chrome deposited on quartz (Advance Reproductions Corp., North Andover, MA) for 5.5 s at 10 mW/cm^2 . Develop the features by immersing in Shipley 351 for 45 s, then rinse with distilled water and dry with a stream of nitrogen. The proper development of the features should be checked under a microscope using a red filter in front of the light source to avoid unwanted exposure of the photoresist. Place the wafers in a desiccator under vacuum for 2 h with a vial (approx 1–2 mL) of (tridecafluoro-1, 1, 2, 2,-tetrahydro-octyl)-1-trichlorosilane (United Chemical Technologies, Bristol, PA).

2.2.2. Molding PDMS Stamp

PDMS (Sylgard 184, Dow Chemical Co.) prepolymer is made by mixing 10 parts of monomer and 1 part of initiator thoroughly in a plastic container and degassing it under vacuum for about 1 h until air bubbles no longer rise to the top. Pour the prepolymer in a Petri dish that contains the patterned silicon wafer, and cure for at least 2 h at 60°C . Peel the PDMS from the wafer and cut the stamps to the desired size with a razor blade.

2.3. Synthesis and Purification of Alkanethiols

The progress of reactions is monitored by thin layer chromatography (TLC) using 0.25-mm silica gel plates (Merck, Mannheim, Germany, or VWR). Column chromatography is performed under nitrogen with silica gel (60–200 mesh, Mallinckrodt). Reactions in nonaqueous solvents are carried out under nitrogen or argon. Organic solvents, unless specified, are HPLC grade (Mallinckrodt, Phillipsburg, NJ). Tetrahydrofuran (THF) that was used as a reaction solvent is distilled freshly on a still that contains benzophenone (1 g/L, Aldrich) and sodium (1 g/L, Aldrich). Dichloromethane used as a reaction solvent is distilled freshly on a still that contains calcium hydride (1 g/L, Aldrich). NMR spectra were collected on samples dissolved in chloroform-*d* (Cambridge Isotope Laboratory). General synthetic procedures are described; the specified quantities of material can be varied by keeping the molar ratios constants to fit the needs of each laboratory. More detailed descriptions of basic organic laboratory techniques are found in Zubrick (25).

2.3.1. Purification of Hexadecanethiol (HDT; 2 mM in ethanol)

Hexadecanethiol (HDT) (Aldrich) is purified by flash chromatography using hexane as the eluant or by distillation at reduced pressure. The major impurity is a disulfide. The R_f of the product is approx 0.4. The typical ^1H NMR spectrum has the following peaks: δ 1.25 (broad singlet, 29 H), 1.6 (quintet, 2 H), 2.5 (quartet, 2 H).

2.3.2. (1-mercaptoundec-11-yl)tri(ethylene glycol) (EGT; 2 mM in ethanol) (26–28)

Reaction mixtures are concentrated by rotary evaporation at reduced pressure. The purification of the final product and of the intermediates is carried out using flash column chromatography with silica gel and 98:2 dichloromethane:methanol as the eluant; typical values of R_f are provided.

2.3.2.1. UNDEC-1-EN-11-YL(TRIETHYLENEGLYCOL)

Mix 0.34 mL (4.3 mmol) of 50% aqueous sodium hydroxide with 3.2 g (21 mmol) of tri(ethylene glycol) (Aldrich) and stir for 30 min in an oil bath at 100°C. Add 1 g (4.3 mmol) of 11-bromoundec-1-ene (Pfaltz and Bauer) and stir at 100°C for 24 h under argon. After cooling, the reaction mixture is extracted six times with hexane (50–100 mL aliquots), and dried with sodium sulfate (Aldrich). Combine the hexane portions, concentrate them, and purify the resulting yellow oil ($R_f=0.3$): a typical yield is approx 70%. ^1H NMR (250 MHz) δ 1.2 (broad singlet, 12 H), 1.55 (quintet, $J = 7$ Hz), 2.0 (quartet, 2 H, $J = 7$ Hz), 2.7 (broad singlet, 1 H), 3.45 (triplet, 2 H, $J = 7$ Hz), 3.5–3.8 (multiplet, 12 H), 4.9–5.05 (multiplet, 2 H), 5.75–5.85 (multiplet, 1 H).

2.3.2.2. [1-[(METHYLCARBONYL)THIO]UNDEC-11YL]TRI(ETHYLENE GLYCOL)

Dissolve 0.6 g (2 mmol) of the previous compound in 20 mL of freshly distilled THF; add 10 mg of recrystallized 2, 2'-azobisisobutyronitrile (Aldrich) and 1.4 mL (20 mmol) of thiolacetic acid (Aldrich) and irradiate for 6–8 h with a 450-W medium-pressure mercury lamp (Ace Glass) filtered through Pyrex. Check that the reaction has reached completion before work-up. Take out a 0.1-mL aliquot, reduce under pressure and take an NMR spectrum. The signal from the protons of the alkene group at $\delta = 4.8\text{--}6$ ppm should disappear if the reaction has gone to completion. Concentrate the reaction mixture and purify ($R_f = 0.3$): a typical yield is approx 80%. ^1H NMR (250 MHz) δ 1.2 (broad singlet, 14 H), 1.6 (multiplet, 4 H), 2.3 (singlet, 3 H), 2.85 (triplet, 2 H, $J = 7$ Hz), 3.45 (triplet, 2 H, $J = 7$ Hz), 3.5–3.75 (multiplet, 12 H).

2.3.2.3. (1-MERCAPTOUNDEC-11-YL)TRI(ETHYLENE GLYCOL)

Dissolve 0.4 g (1 mmol) of the previous compound in 2 mL of freshly distilled dichloromethane and 8 mL of degassed (argon or nitrogen for 30 min) methanol. Add 0.9 mL (1.2 mmol) of 1.3 M sodium methoxide (Aldrich) in degassed methanol. After 45 min, bring the reaction mixture to neutral pH using DL-camphor-10-sulfonic acid (Aldrich), concentrate and purify ($R_f = 0.25$); a typical yield is 50%. ^1H NMR (250 MHz) δ 1.1 (broad singlet, 14 H), 1.2 (triplet, 1 H, $J = 7$ Hz), 1.5 (multiplet, 4H), 2.3 (singlet, 3 H), 2.5 (quartet, 2 H, $J = 7$ Hz), 3.0 (broad singlet, 1 H), 3.4 (triplet, 2 H, $J = 7$ Hz), 3.5–3.75 (multiplet, 12 H).

3. Methods

3.1. Micropattern Stamping Procedure

1. Lay substrate on clean and flat surface, with gold facing upwards. Take care not to scratch the surface with sharp forceps, or by placing substrate upside down. If there is dust visible on the substrate, blow gently with pressurized air or nitrogen.
2. Rinse the PDMS stamp with ethanol and blow off vigorously with a stream of pressurized air or nitrogen for at least 10 s. If any dust remains on the stamp, repeat this procedure.
3. Dip a Q-tip into a 2-mM solution of hexadecanethiol in ethanol and gently paint a layer of the solution onto the PDMS stamp. Use a stream of air or nitrogen to gently evaporate the ethanol off the stamp.
4. Gently place the stamp face down onto the gold-coated substrate. Allow the stamp to adhere. This step may require putting gentle pressure on the stamp to press it against the substrate. Let the fully adhered stamp remain on the substrate for at least 10 s.
5. Using forceps, gently peel away the stamp from the substrate, being certain not to smear the stamp against the substrate or to let the stamp re-adhere to the substrate.

6. Return to **step 2** to continue stamping more substrates. After all substrates are stamped, proceed to **step 7**.
7. Using a Pasteur pipet, deliver a solution of EGT dropwise onto each substrate until the liquid covers it entirely. This usually requires approx 0.5–1 mL per square inch of substrate. Incubate with EGT for 30 min.
8. Using forceps cleaned with ethanol and blown dry, grasp the corner of the substrate and rinse with a stream of ethanol on both sides of the pattern for 20 s. Place the substrate on a clean surface, and rinse the forceps with ethanol. Grasp the substrate again in a different location and rinse again with ethanol to wash the area previously masked by the forceps.
9. Blow the ethanol off the substrate with pressurized air or nitrogen.
10. The stamped substrates should be placed into containers taking care not to allow the patterned surface to rub against coarse surfaces. They are stored under nitrogen gas in a cool, dark location. Place the containers in ziplock bags that are filled with nitrogen.

3.2. Coating Stamped Substrates with ECM Proteins and Plating Cells

1. To coat the substrates with ECM, make a solution of the protein (25 $\mu\text{g}/\text{mL}$) in PBS. Typically, 0.25-mL solution per square inch of substrate is sufficient.
2. Place a 0.25-mL drop of ECM solution onto bacteriological Petri dishes or another disposable hydrophobic surface. Float each substrate, with patterned side face down, onto the drops. Let sit 2 h at room temperature.
3. After 2 h, add a large amount (5–15 mL) of 1% bovine serum albumin (BSA, Fraction V, Sigma, St. Louis, MD) dissolved in PBS directly to dish. Remove substrates and place directly into plating medium (remember to flip slide so pattern is facing up again).
4. Plate cells directly on substrates using standard experimental technique.

4. Notes

1. Gold Slide: Choice of glass: We find No. 2 glass cover slips to be less likely to break than No. 1, although at the same time not too heavy to pick up with forceps. We have successfully used standard histology mounting slides as well.
2. Gold Slides: Evaporation: We recommend using an evaporator rather than a sputter coating system to coat the substrates for several reasons. Most sputterers are single source, and are impractical for coating two different metals (Ti and Au) on a substrate. Sputtering also gives less homogeneous films that would require an additional annealing step to correct. And last, sputtering systems generally produce films with higher quantities of metal oxides and other impurities that would interfere with the generation of the SAM surface.
3. Gold Slides: Storage: Typically, gold-coated substrates become “mottled” after 4–5 wk and are no longer deemed suitable for experiments; streaks with heterogeneous transparency develop (they are obvious to the naked eye). This may be

- caused by rearrangements in the thickness of the gold layer that are related to impurities present on the glass before evaporation of the gold. Gold substrates that are stamped immediately after evaporation are generally more stable over time (about 3 mo) perhaps because the SAM acts as a resist against impurities (29).
4. **Wafers:** Rinse only with water and avoid all contact with organic solvents.
 5. **PDMS Stamp: Pouring and curing:** Often small air bubbles form in the PDMS after it is poured on the master. Cover the dish and gently tap it to allow the bubble to diffuse out of the prepolymer. Typically, stamps are 0.5–1 cm tall.
 6. **PDMS Stamp: Peeling off of silicon:** During curing, a layer of PDMS forms underneath the wafer and holds it to the dish. Invert the dish and gently press on the bottom side of it until the cured PDMS dewets from the surface of the dish. Invert the dish and use a dull edge to trace the contour of the PDMS so as to lift it off the dish. Often the PDMS remains attached to the wafer. Carefully cut the layer of PDMS found under the wafer and gently peel the two surfaces away from each other.
 7. **Thiol Storage:** Typically, alkanethiols that are kept in ethanolic solutions for more than three month become oxidized and form significant amounts of disulfides. Disulfides of EGT are detected by TLC as spots with an R_f of approx 0.15, whereas the thiol has an R_f of 0.25 (using 98:2 CH_2Cl_2 : CH_3OH as the eluant). By NMR, disulfides can be distinguished from alkanethiols by the presence of a triplet of peaks (from the methylene group adjacent to the sulfur atom) at approx 2.6 ppm instead of a quartet at 2.5 ppm. Although disulfides are known to form SAMs with interfacial properties similar to those formed with alkanethiols, their assembly is 75 times slower (30).
 8. **Thiol Stamping: Over and understamping.** Observe the play of light, at an angle, on the micropattern to ensure that the stamp has fully adhered to the substrate. Usually, a pink color will ensure that full adhesion has occurred. Both under and overstamping results in a loss of this interference pattern. Make sure that no patches of nonadhesion remain. Always stamp the hexadecanethiol; stamping the EGT results in less efficient pattern transfer, incomplete formation of SAM, and nonspecific adsorption of proteins in the EGT regions.
 9. **Protein coating:** When adding the BSA solution to bring the substrates out of fibronectin coating, the slides sink onto the dish and adhere to it; since the substrates face the bottom of the dish, the pattern may be damaged. To avoid this, add the BSA solution around the edges of the substrate so that the slides remain afloat.
 10. **Cell culture:** For cell culture, cells should ideally be plated without serum (at least for 1 h), before washing serum media back in. The serum activates certain cell types to attach where they should not. This is cell-type specific, so some exploration may be necessary.
 11. **Cell culture:** The actual surface area that is adhesive on patterned slides is a fraction of that of a regular substrate; therefore, cells should be accordingly seeded at seemingly very low concentrations.

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