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CHAPTER 19

Use of Micropatterned Adhesive Surfaces for Control of Cell Behavior

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I. Introduction

Extracellular matrix (ECM) plays a central role in cell regulation, both *in vivo* during tissue development and in cell culture (Ingber, 1997). Analysis of the molecular basis of cell regulation by ECM has led to the identification of specific transmembrane receptors,

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known as integrins, that can activate many of the same intracellular signaling cascades that are induced when soluble growth factors bind to their own receptors (Clark and Brugge, 1995; Giancotti and Ruoslahti, 1999; Calderwood *et al.,* 2000). However, other studies suggest that the ECM may provide different regulatory signals to the cell depending on the substrate's ability to resist cell tractional (tensional) forces and thereby modulate cell shape (Ingber and Folkman, 1989a; Ingber, 1991). In support of this possibility, many studies have demonstrated a direct correlation between cell spreading and growth (Folkman and Moscona, 1978; O'Neill *et al.,* 1986; Ingber, 1990) and between retraction or rounding and differentiation (Glowacki and Lian, 1987; Watt *et al.,* 1988; Ingber and Folkman, 1989b; Mooney *et al.,* 1992).

Analysis of this structural form of cell regulation has been limited by the availability of defined culture systems. In some of the past *in vitro* studies, cell shape was modulated by overlaying conventional tissue culture dishes with varying amounts or distributions of a nonadhesive blocking polymer (e.g., poly[hydroxyethyl methacrylate]) and then plating the cells in serum-containing medium: in this method, cell rounding is induced as the thickness of the blocking layer is increased (Folkman and Moscona, 1978; O'Neill *et al.,* 1986; Glowacki and Lian, 1987; Watt *et al.,* 1980; Ingber, 1990). In other experiments, cells were cultured in chemically defined medium on otherwise nonadhesive surfaces that were precoated with different densities of ECM molecules, causing cell spreading to increase as the coating density was raised (Ingber and Folkman, 1989b; Ingber, 1990; Mooney *et al.,* 1992). Because serum contains high amounts of ECM proteins, in particular vitronectin and fibronectin, in neither type of experiment was it possible to distinguish signals conveyed by cell distortion from those elicited by differences in integrin (ECM receptor) binding. For this reason, we set out to develop methods to control structural interactions between cells and ECM independently of growth factors or changes in integrin binding, and thereby to make cell distortion an independent variable.

Our approach to cell shape control involved microfabrication of adhesive islands of defined size, shape, and position on the micron scale, surrounded by nonadhesive boundary regions. The surfaces were coated with a saturating density of an ECM molecule and cells were then plated on these surfaces in chemically defined medium containing saturating amounts of recombinant growth factor. Thus, the only variable was the size and shape of the island and hence the degree to which the cell could physically distend. The formation of the islands was accomplished by creating patterns of self-assembled monolayers (SAMs) of alkanethiolates on gold that either support or prevent surface interactions with proteins (Prime and Whitesides, 1991, 1993; Mrksich and Whitesides, 1996). Hydrophobic SAMs rapidly and irreversibly adsorb ECM proteins, and hence promote cell adhesion; SAMs that present polyethylene glycol (PEG) moieties effectively resist protein adsorption, and thus prevent cell adhesion (Prime and Whitesides, 1991, 1993). Our approach was to pattern these two SAMs on a surface with micron-scale resolution to define the pattern of ECM that adsorbs onto the surface, and hence the pattern of cell adhesion and spreading.

Micropatterned culture surfaces (e.g., containing square islands with edge lengths of 5, 10, 20, 30, or 40 μm separated by defined nonadhesive regions; Figs. 1A, 1B) were created using a soft-lithography-based, microcontact printing technique (Singhvi

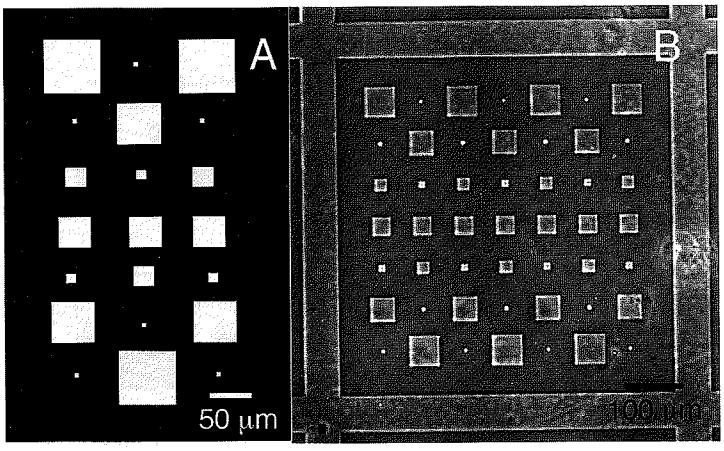

Fig. 1 Creation of a micropatterned adhesive surface using microcontact printing with self-assembled monolayers (SAMs) of alkanethiolates. (A) Drawing of the design for a photomask that includes squares with edge lengths of 5, 10, 20, 30, and 40 μm, separated by varying nonadhesive regions. (B) A phase contrast micrograph of a microfabricated surface containing the pattern shown in A and coated with fibronectin.

et al., 1994; Chen *et al.,* 1997). In this method, the desired pattern is created using computer design software, printed to a mask, and then transferred to a thin film of photoactive polymer (photoresist) on a silicon wafer; standard photolithographic techniques are used in transferring the corresponding pattern onto the layer of photoresist overlaying the wafer, thus creating a "master." A "rubber stamp" containing the imprint (negative form) of the topographical surface on the silicon wafer is then generated by pouring and polymerizing polydimethylsiloxane (PDMS) against the master (Figs. 2A–2D) (Wilbur *et al.,* 1995; Xia *et al.,* 1996; Takayama *et al.,* 2001). The dimensions of these stamps can be as low as 200 nm if necessary and can be designed to exhibit almost any geometric pattern. The stamp is peeled off the master, "inked" with hydrophobic alkanethiols, and then tightly apposed to the surface of a gold-coated cover glass. This results in the transfer of the hydrophobic alkanethiol molecules to the glass surface, which self-assemble into an almost crystalline molecular monolayer that is limited to the regions of the geometric pattern of the islands created on the original master (Figs. 2E–2I) (e.g., 30 μm square island that appeared as an elevated 30 μm square region on the surface of the rubber stamp). Next, a solution containing nonadhesive PEG alkanethiolate, which contains terminal tri(ethylene glycol) groups such as $HS(CH_2)_{11}O(CH_2CH_2O)_2CH_2CH_2OH$, is added to the patterned surface. This alkanethiol self-assembles in the remaining uncoated surfaces between the hydrophobic-SAM covered islands, thereby creating a continuous SAM covering the entire gold-coated area. While the hydrophobic islands support protein adsorption, these intervening PEG-covered barrier regions remain nonadhesive. Thus, when saturating amounts of ECM molecules, such as fibronectin, are added to these SAM-covered surfaces, they adsorb only to the surfaces of the adhesive islands coated with the hydrophobic SAM (Fig. 3).

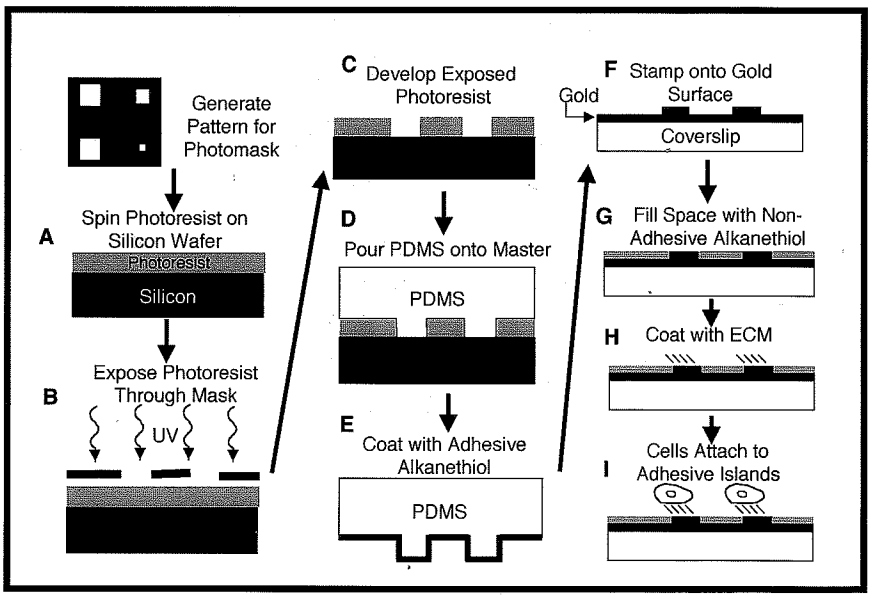

Fig. 2 Schematic of the soft lithography-based microcontact printing method. First, the design for the micropattern is drawn to scale using a commercial drawing package and saved as an electronic file. This file is used to fabricate a photomask with the identical features. (A) A silicon wafer is coated with a thin layer of photoresist. (B) The photomask is overlaid on the wafer and exposed to ultraviolet light thus protecting underlying regions of the photoresist from light exposure. (C) The photoresist is chemically developed resulting in the dissolution of photoresist from the light-exposed regions; the remaining bas-relief pattern retains the same pattern as the photomask. This pattern is exposed to vapors of (tridecafluoro-1,1,2,2,-tetrahydrooctyl)-1-trichlorosilane to reduce its adhesivity to the stamp. (D) Polydimethylsiloxane (PDMS) elastomer is poured over the pattern and cured for at least 2 h at 60°C. When the stamp is cut out and peeled off the original surface, it retains the complementary features of the master silicon wafer. (E) The molded surface of the PDMS stamp is "inked" with the adhesive alkanethiol, which promotes protein adsorption and cell adhesion. (F) The stamp is brought into contact with a gold-coated cover glass for 15 s to transfer the adhesive alkanethiol, which self-assembles into a monolayer in the regions where the stamp contacts the surface. (G) The stamp is removed and the entire surface is covered with a nonadhesive alkanethiol, which coats the remaining exposed regions of the gold surface and resists protein adsorption. (H) When extracellular matrix (ECM) molecules are added to this surface, they only adsorb to the adhesive islands. (I) Cells are seeded onto the cover glass where they attach and spread on these geometrically defined, ECM-coated islands.

Importantly, living cells only adhere to the ECM-coated adhesive islands when they are cultured on these surfaces. We found that when cells adhered to the ECM-coated islands, they spread over the fixed ECM anchors to cover the surface of the island, yet spreading ceased when the cell periphery reached the nonadhesive boundary (Singhvi *et al.,* 1994; Chen *et al.,* 1997, 1998; Huang and Ingber, 1999, 2000). Thus, as a result of the cells exerting tractional forces on their ECM anchors, they changed their morphology and literally "took on the shape of their container" (i.e., the geometric form of their micropatterned island); cells exhibited 90° corners when cultured on square islands and appeared round on circular islands (Figs. 4A–4D). In this manner, the effects

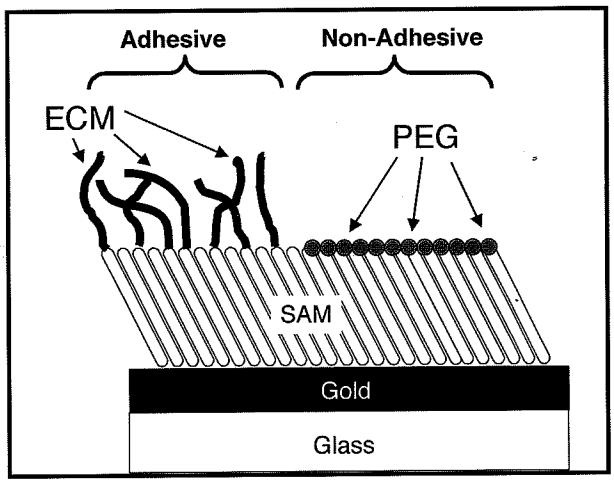

Fig. 3 Schematic representation of the local distribution of adhesive and nonadhesive SAMs of alkanethiolates at the edge of an adhesive island. Molecules such as ECM adsorb to the adhesive SAM (hexadecanethiol) while the regions coated with the nonadhesive SAM polyethylene glycol resist protein attachment.

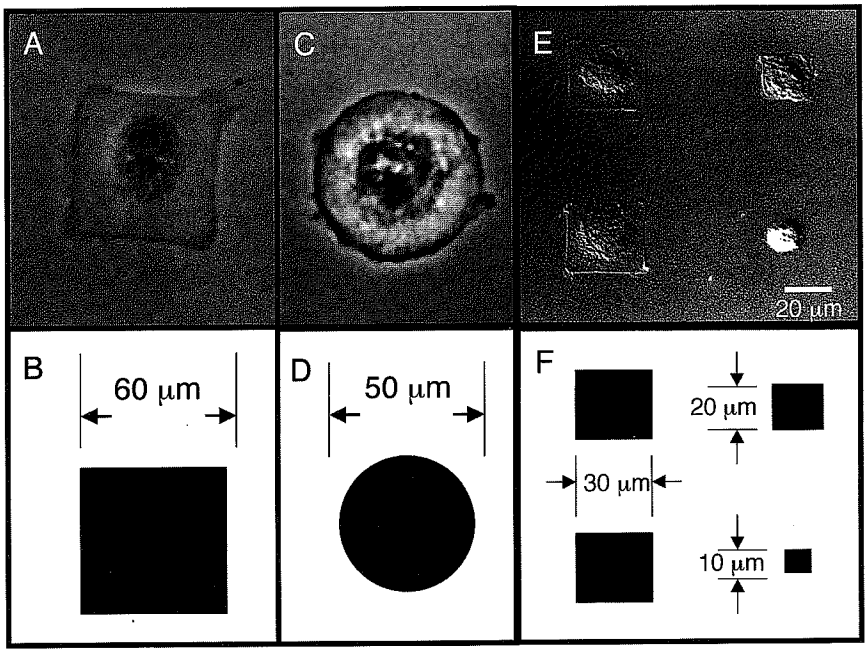

Fig. 4 Microscopic images (A,C,E), and corresponding adhesive island designs (B,D,F), of capillary endothelial cells whose shape and size were controlled using micropatterned adhesive surfaces. Cells cultured on square (A,B,E,F) or circular (C,D) adhesive islands coated with fibronectin. Note that cells may be held in different shapes when adherent to the same ECM density and cultured within the same medium containing saturating amounts of growth factor using this method, as shown in E,F. Scale bar = 20 μm.

of varying cell size and shape on cell functions (e.g., growth, apoptosis, differentiation) could be analyzed independently of both ECM density and soluble growth factors (Figs. 4E, 4F).

When we studied hepatocyte function using this method, we found that cell growth increased in direct proportion as island area was increased and cell spreading was promoted (Singhvi *et al.,* 1994). Conversely, as spreading was prevented and growth was turned off, differentiation (e.g., secretion of liver-specific products, such as albumin) was switched on. Similarly, when we studied capillary endothelial cells, we observed a similar link between spreading and growth (Chen *et al.,* 1997). In these cells, restricting cell spreading to a very high degree (island diameter less than 20 μm) switched on the apoptosis (cellular suicide) program. More recently, we created linear micropatterned adhesive surfaces that restrict capillary cells to a moderate degree of spreading that is not consistent with growth or apoptosis and that permit cell–cell interactions to take place. These capillary cells switch on a differentiation program and undergo capillary tube formation *in vitro* under identical culture conditions (Dike *et al.,* 1999).

One possible caveat in these studies was that the cells contacted more area of ECM (and hence more ECM molecules) on large vs small islands, even though the local ECM molecular coating density was saturating (which should promote optimal integrin clustering and intracellular signaling). However, increasing ECM contact area could increase the total amount of integrin binding or increase accessibility of cells to matrix-bound growth factors, thereby influencing cell behavior through chemical rather than structural means.

To explore this possibility more fully, micropatterned adhesive surfaces were created containing closely spaced adhesive circular islands of either 3 or 5 μm in diameter, to approximate the size of individual focal adhesions. When capillary cells were cultured on these surfaces, individual cell bodies spread across the intervening nonadhesive areas of the surfaces by stretching processes from one small adhesive island to another (Fig. 5). By changing the spacing between adhesive islands, cell spreading could be increased more than 10-fold without significantly altering the total cell-ECM contact area (Chen *et al.,* 1997). On these surfaces, DNA synthesis scaled directly with projected cell area and not with cell–ECM contact area. Apoptosis was similarly turned off by cell spreading, even though the cell–ECM contact area remained constant under these conditions. More recent studies confirm that some forms of integrin receptor signaling are fully activated on the small ECM-coated adhesive islands: for example, cells on small and large islands exhibited identical levels of activation of matrix metalloproteinase-2 in response to matrix binding (Yan *et al.,* 2000). Additional studies have shown that the effects of cell distortion on growth are mediated by specific alterations in the cytoskeleton, including changes in cytoskeletal tension.

Thus, use of this novel microscale patterning technology has revealed that the ECM apparently can convey distinct signals to cells based on its ability to resist cell tractional forces and to promote cell distortion. More importantly, cell shape per se appears to be the critical determinant that switches growth factor-stimulated cells between life and death, and also between proliferation and quiescence. This is critical because local changes in cell–ECM interactions appear to be responsible for establishment of the local growth

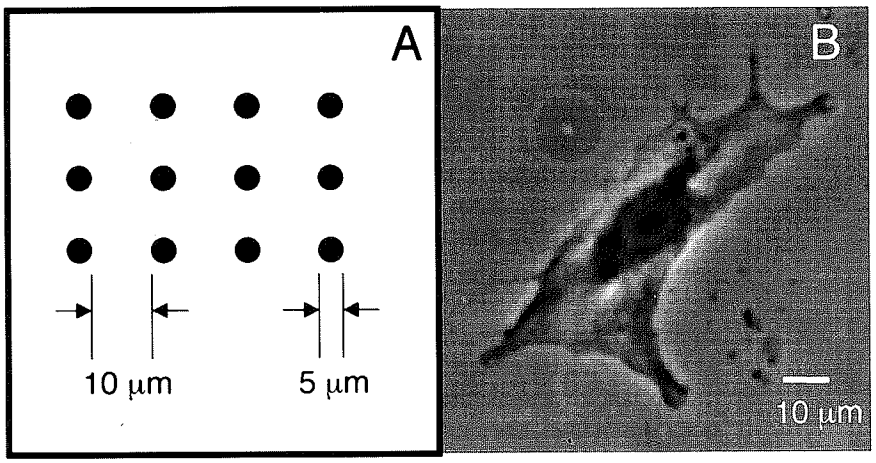

Fig. 5 A cell spread on a micropatterned surface that was used to investigate the effects of cell size and shape independently of cell–ECM contact area. (A) Schematic of the micropattern containing circular adhesive islands that are 5 μm in diameter and separated by 10-μm nonadhesive regions. (B) Phase contrast photograph of a cell that spreads over multiple focal adhesion-sized fibronectin-coated islands as shown in A. Scale bar = 10 μm.

differentials that drive pattern formation during morphogenesis *in vivo* (Banerjee *et al.*, 1977; Huang and Ingber, 1999).

Surfaces with geometrically defined chemistry can be fabricated with techniques other than microcontact printing with SAMs of alkanethiolates, such as vapor deposition and photolithography. In vapor deposition, adhesive metals (e.g., palladium) are deposited onto a surface of nonadhesive polyhydroxyethyl methacrylate, in specific patterns, by delivering them through a removable occlusive mask (e.g., a commercially available electron microscope grid or copper foil with defined pore size, shape, and position) (O'Neill *et al.*, 1986, 1990). Ultraviolet light can also be used with photoreactive chemicals to spatially pattern adhesive and nonadhesive regions. Specifically, photochemical changes in organosilanes are used to create adhesive and nonadhesive regions through deep UV (193 nm) irradiation (Stenger *et al.*, 1992). Photoimmobilization has been used after conjugating oligopeptides with the Arg-Gly-Asp cell-adhesion sequence on a surface of oligo(ethylene glycol) alkanethiolate. The surface was exposed to a UV light or laser beam which modified the molecular density and thus created spatially patterned adhesive and nonadhesive regions for cell attachment (Herbert *et al.*, 1997). Photoactive polymers including phenyl azido-derivatized polymers also have been coated onto an adhesive polymer surface (tissue culture dish) and irradiated through an overlying photomask, and the unreacted components removed to create spatially patterned surfaces for cell attachment (Matsuda and Sugawara, 1995). Another method involved covering photosensitive poly(ethylene terephthalate) films with poly(benzyl *N,N*-diethyldithiocarbamate-*co*-styrene) and irradiating through a photomask; this approach generated polymer stripes with distinct ionic characteristics that inhibit cell adhesion, thus creating spatially defined

regions of varying adhesivity (DeFife *et al.,* 1999). Finally, other forms of microcontact printing can be used to spatially pattern adhesive and nonadhesive regions to investigate cell–ECM interactions. Specifically, microcontact printing is used to spatially pattern avidin molecules onto a polymeric surface such as poly(ethylene terephthalate) through high-affinity biotin–avidin molecular binding, to form an adhesive region for functionally active biotinylated molecules such as biotin-(G)(11)-GRGDS (Patel *et al.,* 2000; Yang and Chilkoti, 2000). Microcontact printing has also been used in conjunction with the self-assembly of fluid lipid bilayers to spatially pattern regions for cell attachment by printing adhesive regions of fibronectin on glass and then immersing the glass in a lipid bilayer solution: this creates a spatially patterned surface for cell–ECM attachment by producing minimally adhesive regions between the fibronectin areas (Kam and Boxer, 2001).

Although these methods are viable approaches for creating patterns of spatially distributed adhesive and nonadhesive regions for cell attachment through cell–ECM interactions, our method has numerous advantages. First, the resolution of molecular patterning in microcontact printing is much lower (down to 200 nm) than for some of the vapor deposition and photoreactive chemical techniques, which are often limited to 5 micrometers or more. One stamp in microcontact printing can be used for at least one hundred repetitions, in contrast to the photoreactive chemical or vapor deposition methods where photomasks or complex irradiation techniques are required for the fabrication of each surface. The nonadhesive regions of our technique use an "inert" region composed of PEG-SAM, instead of utilization of a coating of passive molecules (such as BSA) which degrade over a period of several days. Finally, the time and material costs of fabricating multiple surfaces by our technique are low relative to the alternate techniques.

In summary, our studies suggest that this micropattern-based method for spatial control of cellular attachment by ECM may have important implications for future work on the structural basis of morphogenetic regulation. This system also can be used to investigate a variety of questions involving complex cell behaviors, including cell motility and pattern generation (Brangwynne *et al.,* 2000) as well as differentiation, apoptosis, and growth. Studying these problems is important in the fields of cell biology, bioengineering, drug discovery, and tissue engineering. In this chapter, we provide a detailed description of the methods used for the preparation and use of micropatterned surfaces, coated with the ECM protein fibronectin, for analysis of cell behavior using bovine capillary endothelial cells. This approach may be easily adapted for use with other adherent molecules and cell types by adopting appropriate culture medium.

II. Materials and Instrumentation

A. Creation of the Photomask, Photolithographic Master, and PDMS Stamps

AutoCAD software from Designers' CADD Company, Inc. (Cambridge, MA) was used to create the geometric features of the micropattern. The photomask was custom made by Advance Reproductions Corporation (North Andover, MA) with chrome deposited on quartz for the pattern transfer. Hexamethyldisilazane was from Sigma-Aldrich

(Cat. No. 44109-1) and Shipley 1813 positive photoresist and 351-photoresist developer were from Microchem Corporation (Newton, MA). Silicon wafers in 3- and 5-inch diameters are from Silicon Sense, Incorporated (Nashua, NH). PDMS (Sylgard 184 silicone elastomer kit) was from Dow Chemical Company. All pattern creation was in a class 100 clean room with a photoresist spinner from Headway Research, Inc. (Garland, TX), a Karl Suss (Waterbury Center, VT) mask aligner, and hot plates. An electron-beam evaporator was used to deposit the metals on the glass surface. Desiccators, distilled water, argon gas, and nitrogen gas were purchased from standard scientific distributors.

B. Synthesis of Alkanethiols and Fabrication of Gold-Coated Surfaces

The chemicals necessary for the synthesis of adhesive and nonadhesive alkanethiol SAMs included (tridecafluor-1,1,2,2,-tetrahydrooctyl)-1-trichlorosilane (Cat. No. T2492; United Chemical Technologies), tetrahydrofuran (Cat. No. 43921-5), deuterated chloroform (Cat. No. 15185-8), 11-bromoundec-1-ene (Cat. No. 46764-2), calcium hydride (Cat. No. 21332-2), hexadecanethiol (Cat. No. H7637), sodium (Cat. No. 48374-5), hexanes (Cat. No. 43918-5), sodium hydroxide (Cat. No. 22146-5), benzophenone (Cat. No. 42755-1), dichloromethane (Cat. No. 43922-3), sodium sulfate (Cat. No. 23931-3), recrystallized 2,2′-azobisisobutyronitrile (Cat. No. 44109-0), thiolacetic acid (Cat. No. T30805), sodium methoxide (Cat. No. 16499-2), tri(ethylene glycol) (Cat. No. T5945-5), and DL-camphor-10-sulfonic acid (Cat. No. 14792-3) (all from Sigma-Aldrich). Merck 0.25-mm silica gel plates (Cat. No. 5554/7) were from EM Science, and silica gel with a 60–200 mesh (Cat. No. 6551) from Mallinckrodt. Other common basic laboratory equipment and materials (e.g., thin-layer chromatography, column chromatography, nuclear magnetic resonance, 450-W medium-pressure mercury lamp, ace glass, rotary evaporator) commonly found in chemistry labs (Zubrick, 1992) were also necessary for these studies. Trichloroethylene (Cat. No. 25642-0), methanol (Cat. No. 43919-3), and acetone (Cat. No. 27072-5) were from Sigma-Aldrich. Microscope cover glasses (#2), petri dishes, cotton swabs, and razor blades were from Fisher Scientific. Titanium (Cat. No. 43367-5) and gold (Cat. No. 37316-8) for metal deposition were from Sigma-Aldrich.

C. Pattern Stamping, ECM Coating, and Cell Plating

Phosphate-buffered saline (PBS, Cat. No. BM-220) was from Boston Bioproducts. Fibronectin (Cat. No. 40008A), was from Collaborative Biomedical Products. Bovine serum albumin (Cat. No. 3220-75) was from Intergen. Nalgene Filter systems (0.22 μm) were from Fisher Scientific (Cat. No. 09-761-111). Dulbecco's modified Eagle's medium (DMEM, Cat. No. 11885-084), penicillin, streptomycin, glutamine (Cat. No. 10378-016), and trypsin (25300-054) were from Gibco BRL. Hepes (Cat. No. 50-20577), human high-density lipoprotein (Cat. No. RP-035), bovine calf serum (Cat. No. SH30072.03), and transferrin (Cat. No. T-1283) were from JRH Biosciences, Intracell, Hyclone Laboratories, and Sigma, respectively.

III. Procedures

A. Creation of the Photomask, Photolithographic Master, and PDMS Stamps

The size, shape, and position of the islands and intervening nonadhesive regions within the micropatterns are empirically determined through preliminary studies with each cell type. A starting point is to design one pattern with multiple islands of different shape and size, separated by different-length nonadhesive regions (e.g., square and circular islands from 5 to 100 μm on each side and diameter, respectively, separated by 10- to 200-μm spaces). The geometric design of the pattern is created using the AutoCAD software drawing package and sent electronically to a commercial photomask printing company that fabricates the photomask. This mask is used to create a master by overlaying the photomask on the photoresist-coated silicon wafer. Through exposure to ultraviolet light and developing the photoresist, the pattern of the photomask is transferred to the photoresist. The resulting features of the photoresist can be used as a mold to fabricate the flexible PDMS stamp by pouring and curing the polymer over the surface of the wafer.

1. Creation of the Photomask

a. Steps

i. Select micropattern with desired island size, shape, and position making sure that the total pattern area does not exceed the area of the silicon wafer (either 3- or 5-inch diameter).

ii. Create a file containing the pattern design using AutoCAD; draw pattern in white with black as the background color.

iii. Scale each drawing object to proper dimensions by adjusting the object size (draw a square and then modify the dimensions to 30 μm on each side).

iv. Save as a dxf or dwg file in electronic format for fabrication of the photomask and transfer to a professional high-precision photomasking company (Advance Reproductions Corporation).

v. Obtain mask and store in a clean environment until needed (preferably the clean room).

2. Creation of Photolithographic Master

a. Steps

i. Clean silicon wafers in sonicating bath for 5 min each in trichloroethylene, acetone, and finally methanol.

ii. Bake wafers at 180°C for 10 min.

iii. Spin coat the wafers with 1–2 ml hexamethyldisilazane for 40 s at 4000 rotations per minute (rpm) followed by Shipley 1813 photoresist for 40 s at 4000 rpm for a thickness of 1.3 μm.

iv. Bake the photoresist for 2.5 min at 105°C.

v. Place the mask on top of the wafer in a mask aligner and expose wafer and photomask to UV light for 5.5 s at 10 mW/cm^2.

vi. Place wafers in Shipley 351 developer for 45 s and rinse with distilled water and dry wafer with nitrogen.

vii. Desiccate wafers under vacuum with 1–2 ml of (tridecafluoro-1,1,2,2,-tetrahydro-octyl)-1-trichlorosilane for 2 h.

3. Creation of PDMS Stamps

a. Steps

i. Mix PDMS using a 10:1 monomer to initiator ratio and degas mixture under vacuum for 1 h.

ii. Cover wafer with prepolymer in a petri dish and cure for a minimum of 2 h at 60°C.

iii. Remove PDMS by cutting around outside of petri dish and peeling the stamp from the wafer.

iv. Cut the stamps to the desired sizes with a razor blade preferably to 1.5 cm by 1.5 cm.

B. Synthesis of Alkanethiols and Fabrication of Gold-Coated Surfaces

Before the flexible PDMS stamp can be used, SAMs must be synthesized and the glass cover glass must be coated with a layer of gold. The nonadhesive alkanethiolate, (1-mercaptoundec-11-yl)tri(ethylene glycol), is created by first combining (undec-1-en-1-yl)tri(ethyleneglycol) with sodium hydroxide, tri(ethylene glycol), and 11-bromoundec-1-ene. This product is purified and then used to synthesize {1-[(methylcarbonyl)thio] undec-11yl}tri(ethylene glycol), and eventually the final nonadhesive product. The adhesive alkanethiol, hexadecanethiol, is purchased from a commercial source and purified before use. Finally, a cover glass must be coated sequentially with titanium and gold for the alkanethiolates to attach to the surface.

1. Synthesis of Alkanethiolate SAMs

a. (Undec-1-en-1-yl)tri(ethylene glycol)

Solutions:

i. Tetrahydrofuran (THF): distilled freshly with benzophenone at 1 g/liter and sodium at 1 g/liter.

ii. Dichloromethane: distilled freshly with calcium hydride at 1 g/liter.

Steps:

i. Mix 0.34 ml of 50% aqueous sodium hydroxide with 3.2 g of tri(ethylene glycol).

ii. Stir for 30 min in oil bath at 100°C.

iii. Add 1 g 11-bromoundec-1-ene, stir at 100°C for 24 h under argon, and allow to cool.

iv. Extract six times with 50–100 ml aliquots of hexanes and dry with sodium sulfate.

v. Combine the hexane aliquots, concentrate them, and purify the resulting yellow oil.

vi. The resulting R_f value of the product (undec-1-en-1-yl)tri(ethylene glycol) is 0.3 with 70% yield and NMR at 250 MHz values of 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*).

b. {1-[(Methylcarbonyl)thio]undec-11yl}tri(ethylene glycol)

Steps:

i. Dissolve 0.6 g of (undec-1-en-1-yl)tri(ethylene glycol) into 20 ml of tetrahydrofuran.

ii. Add 10 mg of recrystallized 2,2′-azobisisobutyronitrile along with 1.4 ml of thiolacetic acid.

iii. Irradiate for 6–8 h with 450-W medium pressure mercury lamp.

iv. Remove a 0.1 ml aliquot for NMR, and then purify. The R_f of the resulting {1-[(methylcarbonyl)thio]undec-11yl}tri(ethylene glycol) is 0.3 with a yield of approximately 80%.

v. NMR at 250 MHz is 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*).

c. (1-Mercaptoundec-11-yl)tri(ethylene glycol)

Steps:

i. Degas 8 ml of methanol under argon or nitrogen for 30 min.

ii. Dissolve 0.4 g of {1-[(methylcarbonyl)thio]undec-11yl}tri(ethylene glycol) in 2 ml of freshly distilled dichloromethane and 8 ml of degassed methanol.

iii. Combine 0.9 ml or 1.3 *M* sodium methoxide in the methanol solution.

iv. Use DL-camphor-10-sulfonic acid to neutralize pH in the reaction solution after waiting 45 min.

v. Concentrate and purify (1-mercaptoundec-11-yl)tri(ethylene glycol) to $R_f = 0.25$ with a yield of 50%.

vi. NMR at 250 MHz δ 1.1 (broad singlet, 14*H*), 1.2 (triplet, 1*H*, $J = 7$ Hz), 1.5 (multiplet, 4*H*), 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*). The nonadhesive alkanethiols should be stored under an inert gas at 2–8°C, protected from light, and used within 1 year.

d. Hexadecanethiol

Steps:

i. Hexadecanethiol, which is purchased from commercial sources, is purified through flash chromatography using hexanes or distillation at reduced pressure. The R_f of the product is approximately 0.4 with the typical NMR spectrum peaks at δ 1.25 (broad

singlet, 29*H*), 1.6 (quintet, 2*H*), 2.5 (quartet, 2*H*). The adhesive alkanethiols should be stored at room temperature, protected from light, and used within 1 year.

2. Fabrication of Gold-Coated Surfaces

a. Steps

i. Rinse cover glasses with hexane and ethanol, then dry with nitrogen.

ii. Load cover glasses onto the carousel of an electron-beam evaporator, and reduce pressure in evaporator to less than 1×10^{-6} torr.

iii. Standardize evaporation rates in chamber to 1 Å/s and allow 200–300 Å of metal to evaporate before opening the shutters.

iv. First, evaporate 15 Å of titanium, and then 115 Å of gold onto the cover glasses.

C. Pattern Formation, ECM Coating, and Cell Plating

The gold-coated surfaces can now be manually stamped with the adhesive alkanethiols. After stamping, the intervening spaces are filled with nonadhesive alkanethiols. Next, the ECM is coated onto the adhesive regions. Cells are then plated onto the micropatterned surfaces to study the ECM-dependent control of cell shape and function.

1. Pattern Stamping

a. Steps

i. Rinse both sides of the cover glass with ethanol and blow-dry with nitrogen.

ii. Place the cover glass with the gold-coated side face up on a clean, flat surface and rinse the stamps with ethanol; dry completely with nitrogen.

iii. Immerse the tip of a cotton swab into a 2 m*M* ethanolic solution of hexadecanethiol, then completely wet and cover the surface of the stamp by swabbing the entire surface at least two times; blow dry stamp with nitrogen.

iv. Manually place the patterned side of the stamp down onto the gold-coated side of the cover glass.

v. Gently place firm pressure from one end of stamp to the opposite end to avoid trapping air bubbles under the stamp and allow the stamp to sit on the gold-coated glass for 15 s.

vi. Hold down cover glass by placing tweezers on an exposed edge of the cover glass and then remove stamp by manually peeling the stamp from this edge to the opposite.

vii. Cover the surface of the cover glass with a 2 m*M* ethanolic solution of (1-mercaptoundec-11-yl)tri(ethylene glycol) drop by drop with a Pasteur pipette and incubate with the solution for 45 minutes.

viii. Rinse the cover glass with ethanol on both sides, completely dry with nitrogen, and store the cover glasses with the patterned side face up under nitrogen in 2–8°C; these should be used within 2 weeks.

ix. Rinse the stamp with ethanol, dry with nitrogen, and store with the PDMS stamp face up in a covered petri dish.

2. ECM Coating

a. Solutions

i. 1 *M* sterile phosphate buffered saline (PBS): Dilute 10× PBS in sterile distilled water and pass through a 0.22 μm filter.

ii. ECM solution: Dilute ECM protein in sterile PBS to a concentration that will produce optimal surface coating (e.g., 50 μg/ml).

iii. 1% Bovine serum albumin (BSA) in PBS (1% BSA/PBS) solution: Dissolve 1% w/v BSA into sterile PBS.

b. Steps

i. Pipette a 250 μl droplet of ECM solution onto a sterile bacteriological petri dish.

ii. After flaming tweezers for sterilization purposes, place cover glass with the patterned side face down onto top of the droplet; this will cause the cover glass to float.

iii. Allow cover glass to sit in a humidified chamber at room temperature for 2 h and then pipette 500 μl of PBS under cover glass to raise level of cover glass for easier removal.

iv. After flaming tweezers again, lift cover glass and place face up in a petri dish containing a 1%BSA/PBS solution for 30 min.

v. Rinse cover glass three times in PBS and then add culture medium to cover the coated surface.

3. Cell Plating

a. Solutions

i. Defined medium: Dulbecco's modified Eagle's medium containing 2 m*M* glutamine, 100 μg/ml streptomycin, 100 μg/ml penicillin, 1 ng/ml basic fibroblast growth factor, 10 m*M* Hepes, and 1% BSA.

b. Steps

i. Aspirate medium from adherent monolayers of bovine capillary endothelial cells or other cell types cultured in sterile tissue culture dishes.

ii. Add 2 ml of PBS with calcium to the monolayer to cover the surface of the cells.

iii. Aspirate PBS and add 0.3 ml of trypsin in a 35 mm dish for 2 min, or until visual confirmation of rounding and detachment from dish.

iv. Using 10 ml of medium containing 1% BSA, pipette cells off surface of dish and collect; centrifuge solution for 5 min at 1000 rpm, aspirate supernatant, and resuspend cells in 5 ml of defined medium with 1% BSA (trypsin inhibitors can be added).

v. After determining cell density, add 10,000 cells per cover glass and then allow cells to attach and spread.

IV. Comments

The SAM and microcontact printing technique presented here creates adhesive islands surrounded by nonadhesive regions and leads to the adsorption of proteins onto the gold-coated surface in geometrically defined patterns. The use of the microcontact printing technique obviates the need for a dust-controlled laboratory environment after fabrication of the master. This technique also reduces the cost significantly compared to reproducing patterned surfaces for each cover glass using standard photolithographic techniques. It also permits larger scale production of the surfaces, because the photolithographic processing step is only used once during the fabrication process, for the initial fabrication of the master.

When drawing the original pattern designs for the mask, a 5-inch silicon wafer should have a total design area only 4.5 inches in diameter, a 3-inch wafer should not use more than 2.75 inches. The use of number 2 cover glass vs number 1 reduces the chance of brittle fracture of the coated surface. Evaporation of metals onto these cover glasses is accomplished using an electron beam evaporator, because sputtering causes inconsistencies in the layer of deposited metal. Each of the synthesized chemical products is concentrated through rotary evaporation at reduced pressure. Thin layer chromatography with 0.25-mm silica gel plates is then used to examine the progress of the reactions: the column chromatography is carried out using silica gel with 60–200 mesh under air, nitrogen, or argon conditions.

V. Pitfalls

A. Surfaces that contain adhesive islands separated by large distances can be compromised because the intermediate areas of the PDMS stamp sag and come into contact with the cover glass. This will force the stamp to print in regions intended to be inert, as the adhesive alkanethiols become deposited in these barrier regions.

B. Stamping of the surfaces is accomplished not just by placement of the stamps on the surface, but also by the application of firm pressure on the stamp. When applying this pressure, work from one corner of the stamp to the opposite corner to avoid trapping air bubbles between the stamp and the surface.

C. After coating the surfaces with ECM, be careful to avoid contact of the printed surface with other surfaces, or drying of the ECM, which can diminish the stability of the printed surface.

D. The conditions for cell culture should be optimized to minimize the presence of ECM proteins in the medium (preferably by removing or minimizing serum), which could alter the cell–matrix interactions. When seeding cells on the coated surfaces we use a density of 10,000 cells/cm^2 for our bovine capillary endothelial cells, although every cell type should be optimized for plating density. High densities can cause monolayers to form that extend from island to island over the entire surface, whereas low densities will cover printed regions sparsely.

E. Keep the gold-coated cover glass in enclosed containers after the initial electron beam deposition and clean with ethanol to prevent dust and other contaminants from adhering to the surfaces. These impurities will cause problems in stamping and in nonspecific attachment of cells to the final surfaces.

F. For deeper feature sizes, SU-8 negative photoresist from Shipley can be used as an alternative to positive photoresist, although a negative of the initial mask must then be utilized.

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