Patterning Mammalian Cells Using Elastomeric Membranes

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We describe the patterning of proteins and cells onto the surfaces of bacteriological Petri dishes, glass, and poly(dimethylsiloxane) (PDMS) with the use of elastomeric lift-off membranes—free-standing polymer films that have circular or square holes with diameters, sides, and height ≥50 μm. Cells are patterned within the physical constraints provided by the holes of the membranes; these constraints can be released to allow the cells to spread onto the rest of the surface or to remain in the pattern by controlling the properties of the surfaces. Careful control of the properties of the surfaces of the substrates are required to cause the cells to adhere to the substrate and not to the membrane, and to avoid damage to the cells on removing the membrane. This strategy of membrane-based patterning—given the acronym MEMPAT for brevity—offers a more convenient way for patterning cells on surfaces and for studying cell spreading than existing methods.

Introduction

This paper describes the use of elastomeric membranes (Figure 1) to pattern the attachment of bovine capillary endothelial (BCE) cells to surfaces that are commonly used in eucaryotic cell culture (plastics, glass). This procedure provides a simple and inexpensive method to grow attached cells within patterned constraints, and then to release the constraints to allow the cells to spread. We call this method "membrane-based patterning", or MEM-PAT, for brevity. We suggest that MEMPAT will be especially useful in studies of spreading and migration that rely on the ability of cells to spread on surfaces from a pattern. Most current techniques for patterning proteins and cells are not directly compatible with a process that requires the cells to be grown within patterned constraints, and then releasing those constraints and allowing the cells to migrate. MEMPAT offers a convenient, versatile, and inexpensive method for patterning cells on surfaces, and for studying cell spreading.

Background. Patterning of cells is an experimental tool that is broadly useful in studying and controlling the behavior of anchorage-dependent cells.[1−4] It is also relevant to applied cell biology, biosensors, high-through-put screening, and tissue engineering.[1,5−15] A set of

techniques that we have developed—soft lithography — provides one set of methods for patterning surfaces and fabricating structures with dimensions in the 1−100 μm range in ways that are useful in cell biology and biochemistry.[16−20] Microcontact printing is particularly versatile as a method for generating patterns of proteins and cells, by patterning self-assembled monolayers (SAMs) of alkanethiolates on the surface of gold.[1,4,21−23] Using μCP, it is straightforward to generate patterns of adhesive proteins (e.g., fibronectin, laminin, vitronectin) on surfaces; these areas of adsorbed protein allow the selective attachment of cells. Although μCP is an experimentally convenient technique that has sufficient resolution to allow

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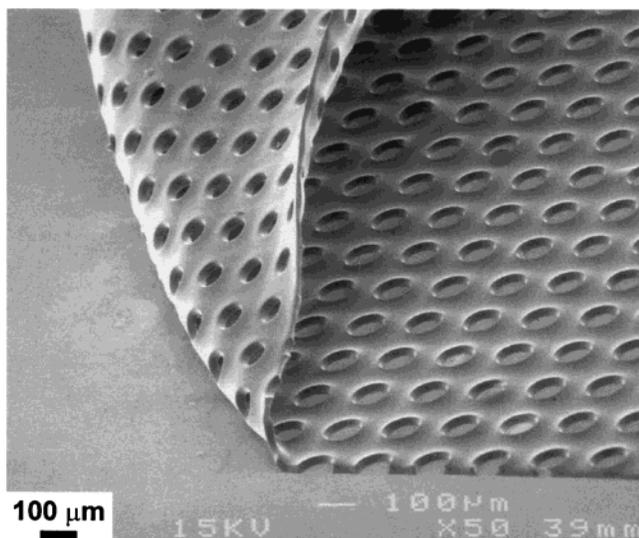(23) Mrksich, M.; Whitesides, G. M. *Trends Biotech.* **1995,** *13,* 228−235.

Figure 1. Scanning electron micrograph of a membrane with 100 μm circular holes; this membrane is ca. 50 μm thick. A part of the membrane was lifted out of contact with the substrate (a Petri dish) to illustrate its flexibility. The edges of the holes appear brighter than the surface of the membrane as a consequence of the curvature of the polymer near the holes (see text for details).

the patterning of single cells, in its simplest configuration it does not allow the cells to be "released" from the pattern; that is, once a pattern of SAMs has been formed, fibronectin adsorbed, and cells attached, there is no practical way of changing the pattern or allowing the cells to spread beyond the boundaries of this pattern. Mrksich and co-workers have recently overcome this limitation of μCP elegantly by partitioning the gold substrate into regions patterned with a hydrophobic alkanethiolate and another alkanethiolate that presents small percentages of an electrochemically active terminal group.[24] After cells attached and spread on the hydrophobic pattern, application of a short voltage pulse changed the oxidation state and polarity of the terminal redox center. This change allowed groups presenting peptide sequences to react with the surface to generate a surface that the patterned cells can spread on. This method requires the synthesis of electroactive alkanethiols, and also requires electrochemical instrumentation; it is thus experimentally more complex than MEMPAT, although it offers a degree of chemical control that MEMPAT does not.

In the past, cell spreading has been studied with techniques that allow *large groups of cells* to be constrained before releasing the constraints to let the cells spread. Pratt et al. clamped a sheet of Teflon that presented circular holes with a diameter of ca. 2 mm to a fibronectin-coated substrate; after the cells had spread inside this area, the Teflon sheet was removed to allow the cells to spread.[25] The method developed by Pratt et al. does not allow control over the size and shape of *individual* cells (as MEMPAT does) and it does not allow patterning a surface with two different types of proteins. Hirschi et al. modified the method of Pratt et al. and generated cocultures of different types of cells; cells were seeded in wells carved into an agarose gel to study cell migration and cell—cell interactions.[26] Several other groups have

used porous agarose slabs that present wells (>2 mm in diameter) to measure the migration of cells such as leukocytes out of the wells in response to the presence of other cells or substances in neighboring wells.[27−30]

We have also developed laminar flow of adjacent fluid streams with low Reynolds numbers (Re < 2000) (FLO) in 100−300 μm scale channels[31,32] to accomplish the patterning of[31] (i) the substrates to which the cells attach, (ii) the cells, and (iii) the surfaces of the cell membranes, all with resolution down to the subcellular level. FLO is straightforward, but restricted to simple patterning, and it has not been explored for patterning the shape and size of cells, or for experiments in which the pattern must change at some point during the experiment. A recent application of soft lithography makes it possible to generate 3D microfluidic networks that are better suited than MEMPAT to generate arbitrary and discontinuous patterns of proteins and cells.[33] The fabrication of these networks requires, however, more steps than the fabrication of membranes.[33,34]

Photolithography can also be used to pattern cells on silicon substrates by patterning alkylsilanes. Although the technology of photolithography is very highly developed, it is not well-suited for applications in cell biology, and especially to applications that require cells to be patterned and then released from the pattern. Photolithography requires the use of clean-room facilities. It does not allow the level of control over the molecular properties of a surface required for the most sophisticated cell-biological experiments.[5,9,35−38] It also does not allow the properties of the surface to be changed during the experiment.

We wished to develop a technique for patterning cells that allowed several types of manipulations to be accomplished easily. (1) We wished to pattern the attachment of mammalian cells conveniently on materials commonly used used in cell culture: polystyrene, glass, polycarbonate. (2) We wished to be able to study cell spreading by allowing the cells to grow to confluence within constraints and then releasing these constraints. We wished to accomplish this task without requiring complex organic synthesis. (3) We wished to retain the convenience and flexibility of soft lithography. We believe that MEMPAT meets these objectives and that it can be applied conveniently and inexpensively both to the patterning of proteins and cells, and to the study of cell spreading.

Strategy. We adapted the idea of "lift-off" from the

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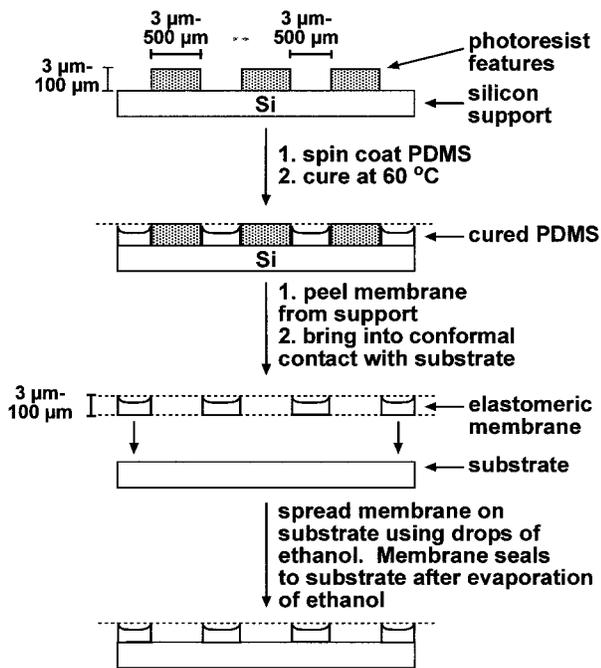
Figure 2. Schematic illustration for the fabrication of membranes. PDMS prepolymer was poured on a silicon wafer that supported features of photoresist. The wafer was spin coated with PDMS to generate a film that was thinner than the height of the features; the prepolymer wets the edges of the features to generate a meniscus. The membrane was then placed in an oven at 60 °C for 2 h. The cured membrane was removed from the photoresist master and brought into conformal contact with a bacteriological Petri dish.

fabrication of microelectronic devices to the patterning of proteins and cells. Lift-off is a process in which a material (typically a metal) is deposited on the surface of a substrate (usually from the vapor phase) that has been patterned with a layer of photoresist. The material deposits through the holes in the photoresist. The photoresist is then dissolved; its dissolution releases the material deposited on it, and leaves a pattern of the material on the substrate; that is, dissolution of the photoresist "lifts off" the layer of material that was deposited on it. In MEMPAT, we replace photoresist with a prefabricated, elastomeric membrane that we refer to as a "lift-off membrane" (Figures 1-2),[39,40] and we use solutions of proteins instead of metals to pattern the exposed surface through the holes in the membrane. This membrane allows patterning the physical access of solutions and suspensions to the surface of the substrate (Figure 3). MEMPAT provides a method to control the location of cellular attachment to surfaces; accomplishing this result requires careful control of the properties of both the membrane and of the surface on which it rests. In this work, we accomplish this control by the adsorption of proteins that direct or prevent the adhesion of cells.

Here, we describe two methods of using MEMPAT. In the first, we pattern the size and shape of cells on surfaces; in the second, we form local patterns of cells in the holes of the membrane, and peel off the membrane to allow the cells to spread from this initial pattern. The major technical differences between the two approaches are the following: the ways in which the membranes are functionalized, and
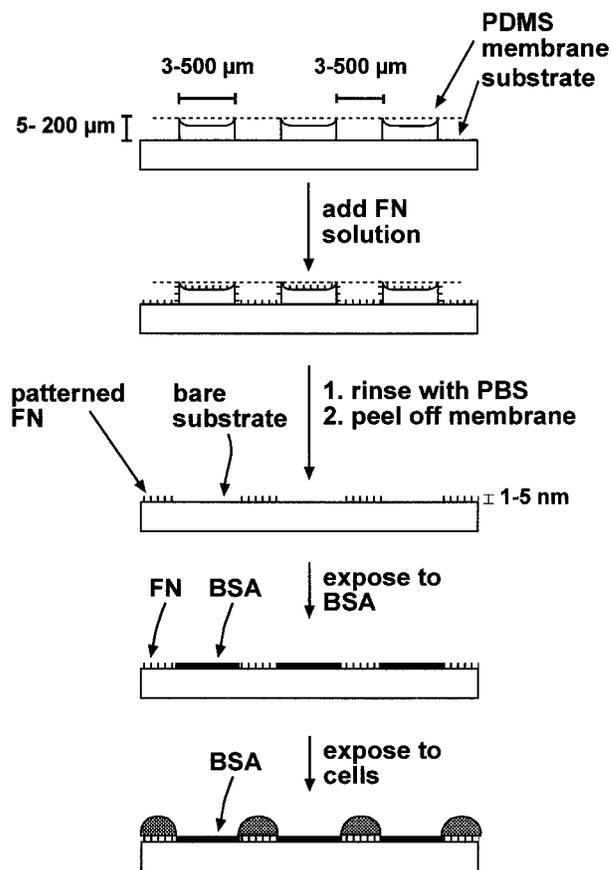
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Figure 3. Schematic illustration of the use of MEMPAT to pattern proteins and cells onto tissue culture dishes. See text for details. The membrane was used to pattern the adsorption of fibronectin (FN, an extracellular matrix protein) to the surface of the substrate. FN adsorbed only to the surface of the substrate that was exposed by the patterned holes in the membranes (Figure 5). Removal of the membrane from the surface generated a pattern of FN. The substrate was then exposed to culture medium that contained bovine serum albumin (BSA) to ensure that the remainder of the surface was coated with a protein that resisted the attachment of cells. Cells from a suspension adhered to this substrate only in the pattern defined by the holes in the membrane (Figure 5). The schematic is not drawn to scale to simplify the representation and it is not meant to suggest that the adsorbed layers of BSA and FN have the same thickness. The features of the membranes have curved lines that result from menisci that form during the spin-coating procedure (see text for details).

the order in which the membrane was removed and the cells attached to the substrate.

MEMPAT for Patterning Proteins and Cells. Use of the membrane to pattern the adsorption of the extracellular matrix protein fibronectin (FN) or gelatin to the surface of a substrate is straightforward (Figure 3). Solutions of FN or gelatin are placed on the assembly of the membrane and the underlying substrate; the protein adsorbs to the exposed surfaces. The removal of the membrane generates a pattern of protein on the substrate; cells adhere selectively to this pattern.

MEMPAT for Studies of Cell Spreading. We first patterned FN or gelatin on a substrate with a membrane in which the top and sides of the features of the membrane had been coated selectively with BSA (Figure 4). The BSA-coated membrane resisted the adsorption of protein *and* the attachment of cells. The cells attached to the areas of the substrate that were coated with protein through the holes of the membrane. They spread to form a layer of cells (which may contain one to a few tens of cells

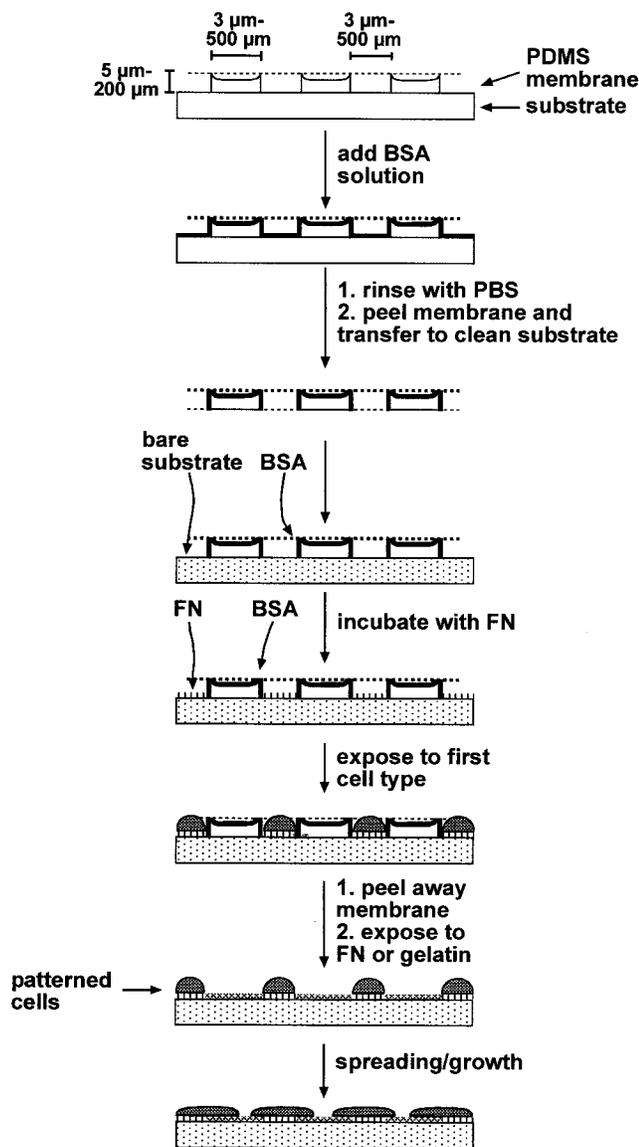　　　　　　　　　　　　　　　　　　　　　　　　　　*Ostuni et al.*

Figure 4. Schematic diagram that describes the use of MEMPAT for studying cell spreading. See text for details. In this procedure, the membrane coated with BSA on one of its sides was used as a mask during the adsorption of FN to a clean surface. Cells adhered to the surface of the substrate that was coated with FN and avoided the walls of the membrane that had been coated with BSA. Upon peeling, the membrane did not damage the cells that remained attached to the surface of the substrate in the pattern defined by the holes of the membrane (Figure 8). The protected areas of the substrate could then be modified by the adsorption of an adhesive protein that allows the patterned cells to spread. As in Figure 3, the dimensions of the cells, the membrane, and the layers of proteins are not drawn to scale for simplicity.

depending on the size of the hole and the density of the suspension of cells) and remained confined to that space until the membrane was removed from the surface. Removal of the membrane from this surface resulted in a pattern of cells on the surface; it also exposed the area of the substrate that had been protected by the membrane to the solution. These regions then adsorbed adhesive protein (which may be the same protein as the first or a different one); after this step, the cells spread across the entire surface from the initial constrained area (Figure 4). This approach could also be used to pattern a second type of cells, instead of studying cell spreading.

Results and Discussion

Fabrication of Membranes. The shapes of the features of the membranes are controlled strictly by the pattern that is generated photolithographically. Jackman et al. have reported that it is usually impractical to generate membranes that are discontinuous or that have features that are not arranged in a regular pattern. Membranes can be used to generate complex patterns on surfaces through the use of sequential lift-off steps.[40]

A pattern of raised features of photoresist was generated using conventional procedures.[40] The masks for the photolithographic step were in general made of a transparency film with features printed on a high resolution printer; for circular features we used a chrome mask, because it was available. We used the transparency masks to generate the required structures quickly and inexpensively using a technique we have described previously as "rapid prototyping".[16,17] The edge resolution of features generated by rapid prototyping is <5 μm for linear features; this limit to resolution is not a constraint for patterning cells, since the features required are typically ≥ 50 μm.

The membranes were fabricated by spin coating PDMS prepolymer onto a silicon wafer having raised features of photoresist to a thickness less than that of these features (Figures 1–2). The thickness of the membranes is determined by the height of the features on the photolithographic master, and by the speed at which PDMS is spin coated. The PDMS wets the walls of the features; this wetting gives rise to menisci around each feature and the top surface of the membranes is not flat.[40] Menisci can be seen around the holes of the membrane in Figure 1. The curvature of the membranes is not a problem for the applications of MEMPAT in cell biology that we will describe; although the adhesion of cells to the membranes will be useful in certain types of experiments, it is not a feature we use here.

After curing the membranes at 60 °C for 2 h, we applied (or "painted") a thicker layer of PDMS prepolymer to the edges of the membranes and cured them. The painted region of the membranes served as a reinforcement layer that made it easier to pick up the membranes without damaging them. We normally used membranes with thickness ≥ 50 μm; these membranes were generally preferable to thinner ones because they were more robust mechanically and less likely to tear than thinner membranes—Jackman et al. have, however, reported the fabrication of membranes with thickness of 5 μm.[40]

We prepared the membranes for biological experiments by extracting low molecular weight organic substances from them by soaking in dichloromethane overnight, and then drying at 60 °C overnight. The membranes were then placed onto Petri dishes, and covered with a few drops of absolute ethanol. This liquid helped to decrease the tendency of the membranes to adhere to themselves and it facilitated the formation of conformal contact between the membrane and the surface of the substrate.[40] The use of ethanol to seal the membrane against the surface also sterilized the membranes and the surfaces of the substrates; ethanol washes are commonly used to sterilize substrates in biological experiments.

The thicker membranes (>50 μm) also had the advantage that they could be reused after experiments involving protein and cell patterning. To reuse them, we washed the membranes with detergent (sodium dodecyl sulfate) and extracted them with dichloromethane. We used thick membranes (>50 μm), because they were less likely to tear than thin ones (<50 μm) during washing.
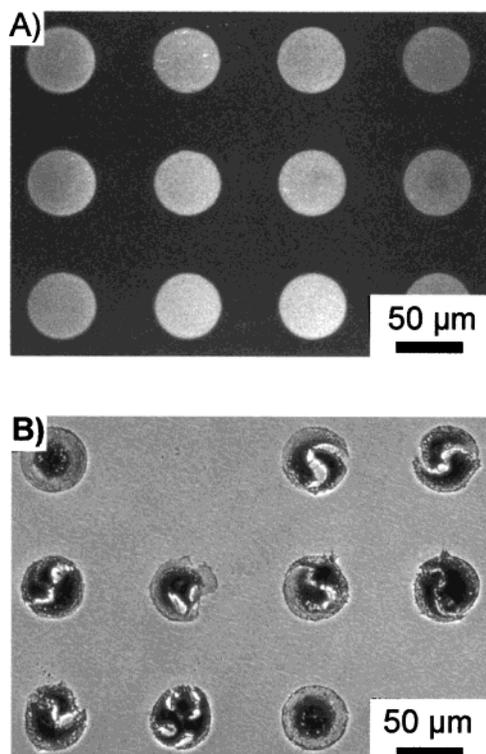
Figure 5. (A) A pattern of FN was generated on a bacteriological Petri dish using MEMPAT. A membrane was incubated with a solution of FN for 1 h following the application and release of vacuum for 30 s. After three rinsing steps with PBS, the membrane was removed from the substrate in the presence of culture medium that contained BSA (Figure 3). The FN pattern on the surface was incubated with fluorescently labeled antibodies that made the FN appear light gray in fluorescence microscopy (Experimental Section). (B) A pattern of cells adhered to circular islands of FN that were prepared using the same method described in (A).

Preparation of the Surfaces: Substrate and Membrane. When MEMPAT was used to pattern proteins and cells, the membrane was not treated; it was used strictly to limit the areas accessible for the adsorption of proteins on the surface of the substrate (Figure 2). In MEMPAT, it is necessary that solutions of proteins enter the holes of the membranes to coat their inner surfaces and the surfaces of the substrates. Buffered solutions of proteins did not wet the low free-energy surface of PDMS; drops of buffer beaded on the surface, and liquid did not enter the holes of the membranes. To force the liquid into the holes, we applied and then released low vacuum (ca. 500 mTorr) several times to remove air bubbles that were trapped inside the holes of the membranes.[41] The membranes were peeled from the substrate after allowing the protein to adsorb for ca. 1 h. After removal of the membrane, the areas of the substrate that had been protected were coated with BSA to resist the adhesion of cells. Figure 5A shows a pattern of FN that was generated following this procedure. We have used MEMPAT successfully to pattern proteins and cells on the surfaces of polystyrene, glass, PDMS, and Si(100)/SiO$_2$; we do not believe that the different characteristics of the substrates affected the properties and the responses of the BCE cells. For convenience, however, we used bacteriological Petri dishes as the substrates for all the experiments that we present in this paper.

(41) Ostuni, E.; Chen, C. S.; Ingber, D. E.; Whitesides, G. M. Unpublished results.

When using MEMPAT to pattern cells for studies of cell spreading, the surface and the edges of the features of the membrane were treated with BSA to prevent cells from attaching to it; the bottom of the membrane was left untreated. We allowed BSA to adsorb to the membrane while it was in contact with a surface. This contact protected the bottom face of the membrane. A layer of BSA adsorbed to the bottom of the membrane prevented it from making conformal contact with the surface of the substrate and solutions of proteins spread over the entire surface of the substrate instead of being localized to the holes of the membranes. We coated the surfaces with BSA, by applying a solution of the protein and applying and releasing vacuum to remove the air bubbles that were trapped in the holes of the membranes. This step also coated the surface of the substrate with BSA and rendered it nonadhesive to cells. The membranes were then picked up with tweezers and moved to a clean substrate; this substrate, with the membrane in place, could then be patterned with fibronectin or gelatin. A key step in moving the membranes is the formation of a conformal seal with the new substrate; we found that this seal was formed best in the presence of PBS buffer (protein-free) that allowed us to position the membranes while maintaining the hydration of the layer of adsorbed BSA—a layer of BSA that has been allowed to dry does not resist the attachment of cells as well as one that has been kept hydrated.

In patterning the adsorption of FN or gelatin to the surface of the clean substrate, we also applied and released vacuum to ensure the removal of all air bubbles from the holes of the membranes. This last patterning step generated a contoured substrate composed of a BSA-coated membrane that resisted the attachment of proteins and cells, and substrate patterned with FN in the holes of the membrane; the adhesion of cells was selective for the FN-coated regions. The membrane could be removed from the substrate to expose the rest of the surface to either a solution of BSA, to leave cells in a pattern (Figure 6), or a solution of gelatin, to allow cells to spread from the FN-patterned surface (that is, the part of the surface originally exposed through the holes in the membrane) onto the rest of the surface.

Removing the Membrane. Proteins were patterned with MEMPAT by simply placing a solution of protein on the membrane for a period of time and then removing the membrane. The excess solution containing the protein was removed by washing, and the membrane was peeled from the substrate with tweezers. We used this procedure to generate a pattern of adhesive protein on the surface that defined the size and shape of the cells that attached to it (Figure 5A).

To study cell spreading with MEMPAT, the membrane was removed from the substrate *after* the cells attached and spread on the islands defined by the holes of the membrane. FN or gelatin were patterned on the substrates through BSA-coated membranes. Cells from a suspension (same conditions as described above) adhered to the FN-coated areas inside the holes of the membranes. After allowing the cells to spread onto the islands for 6–20 h, the membrane was removed with tweezers in the presence of a solution of gelatin (1% w/v in culture medium free of BSA).

Cell Attachment. The first step in patterning cells with MEMPAT was the adsorption of FN or gelatin to the substrate in a pattern defined by the holes of the membrane. Upon removing the membrane, the substrates were exposed to suspensions of BCE cells; we typically used 2 mL of a suspension of 25000 cells/mL in a dish with
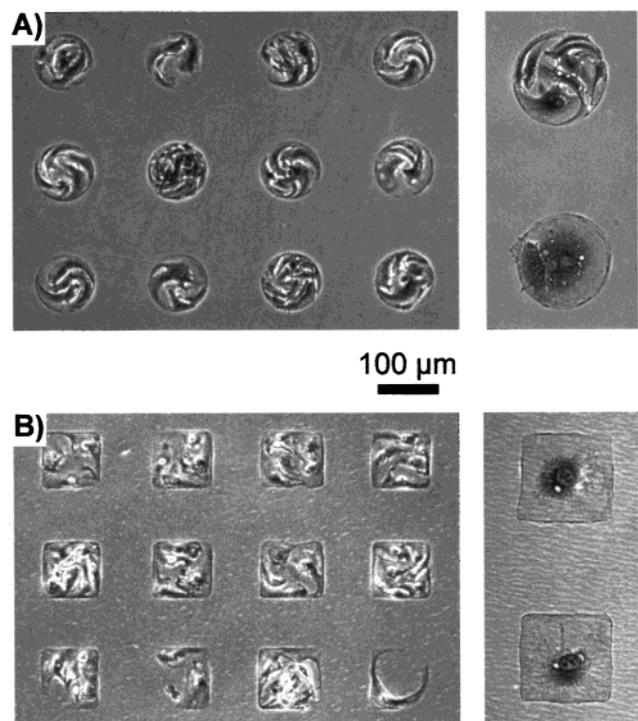
Figure 6. Optical micrographs of BCE cells patterned on a bacteriological Petri dish that presented islands of FN generated using MEMPAT (Figure 3). Details of the procedures used are described in the text and in the Experimental Section. The surface of the membrane and the walls of its holes were coated with BSA. The membrane was placed on a clean bacteriological Petri dish and exposed to a solution of FN (50 μg/mL in PBS) following the procedure described in Figure 3. The membrane and the substrate were covered with a suspension of cells for 24 h. The membrane was removed and the cells were fixed and stained to show the nuclei and parts of the cytoskeleton. (A) Cells patterned on circular islands 100 μm in diameter. (B) Cells patterned on square islands with 100 μm sides. The insets show that a single cell could also cover the entire available surface.

area of 960 mm². Cells adhered to the substrate in a pattern through the holes of the membrane (Figure 5B). The cells were typically allowed to spread on the substrates for 20–24 h (Figure 5B). The use of a hydrophobic PDMS membranes to pattern FN or gelatin (Figure 3) did not allow us to pattern cells by removing the membrane after allowing cells to adhere to the substrate. The procedure described in Figure 3 coated both the membrane and the substrate with a layer of adhesive protein. As a result, cells that were exposed to these substrates adhered both to the surface of the substrate and to the exposed surfaces (top, and walls of the holes) of the membrane (Figure 7A). The cells on the top of the membrane were not a concern, since they were removed with the membrane. Those that attached to both the substrate and the wall/top of the membrane were, however, damaged when the membrane was removed.

To avoid damage to the cells on removing the membrane (for example, when studying cell spreading) it was necessary to pattern proteins and cells in the presence of a membrane that was inert to the adsorption of FN or gelatin and to the adhesion of cells (Figure 4). We coated the membrane with BSA before placing it on a clean substrate and placing a solution of FN or gelatin on it. FN or gelatin adsorbed on the surface of the substrate that was exposed thorugh the holes of the membrane. The layer of BSA adsorbed on the membrane resisted the adsorption of FN or gelatin, and therefore prevented the attachment
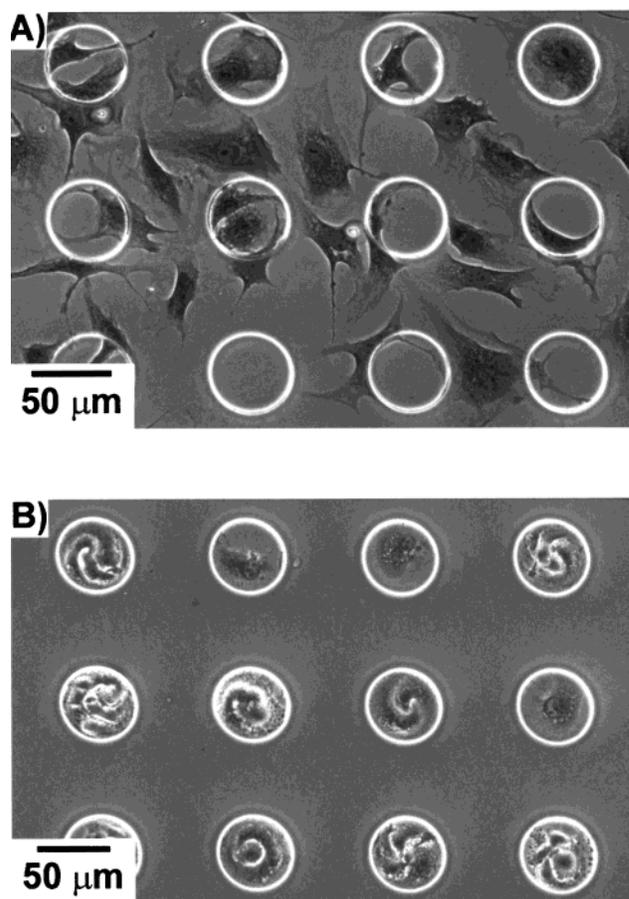

Figure 7. Coating the membranes with different proteins determined whether BCE cells attached to the substrate and the membranes or only to the substrate. (A) Cells adhered to the entire assembly of membrane and substrate when both were coated with FN using MEMPAT and the procedure summarized in Figure 3. (B) Cells adhered selectively to the surface of the substrate that was coated with FN using a membrane in which the top surface and the sides of the holes were coated with BSA (Figure 4). The cells did not attach to the membrane. These images were obtained with membranes that were 5 μm thick in order to facilitate focusing on the cells at the bottom of the holes. Such thin membranes could be stretched easily; hence, some of the holes appear distorted.

of cells; thus, cells attached only to the parts of the substrates that were coated with FN or gelatin in the presence of the membrane (Figure 7 B). The culture medium contained a high concentration of BSA (1% w/v) to ensure that the adsorbed BSA did not exchange with other adhesive proteins in the medium; hence, the surfaces remained resistant to cell adhesion for at least 48 h.

Assessing Damage to Cells Caused by the Peeling Step. The removal of the membrane from the surface of the patterned substrate had the potential to damage the membranes of cells that were simultaneously attached to both the PDMS membrane *and* the substrate. We tested the integrity of the cell membranes using a fluorescence assay after peeling off the elastomeric membrane. After removal of the membranes, we incubated the cells with a solution of propidium iodide (PI) – a dye that diffuses only into cells that have damaged membranes and that becomes more fluorescent upon complexing with DNA. Figure 8 (A and C) shows that removing BSA-coated PDMS membranes from a substrate does not damage the membranes of the cells that are attached on the areas of the substrate which were exposed by the elastomeric membranes. PI was incorporated in ≤3% of cells after
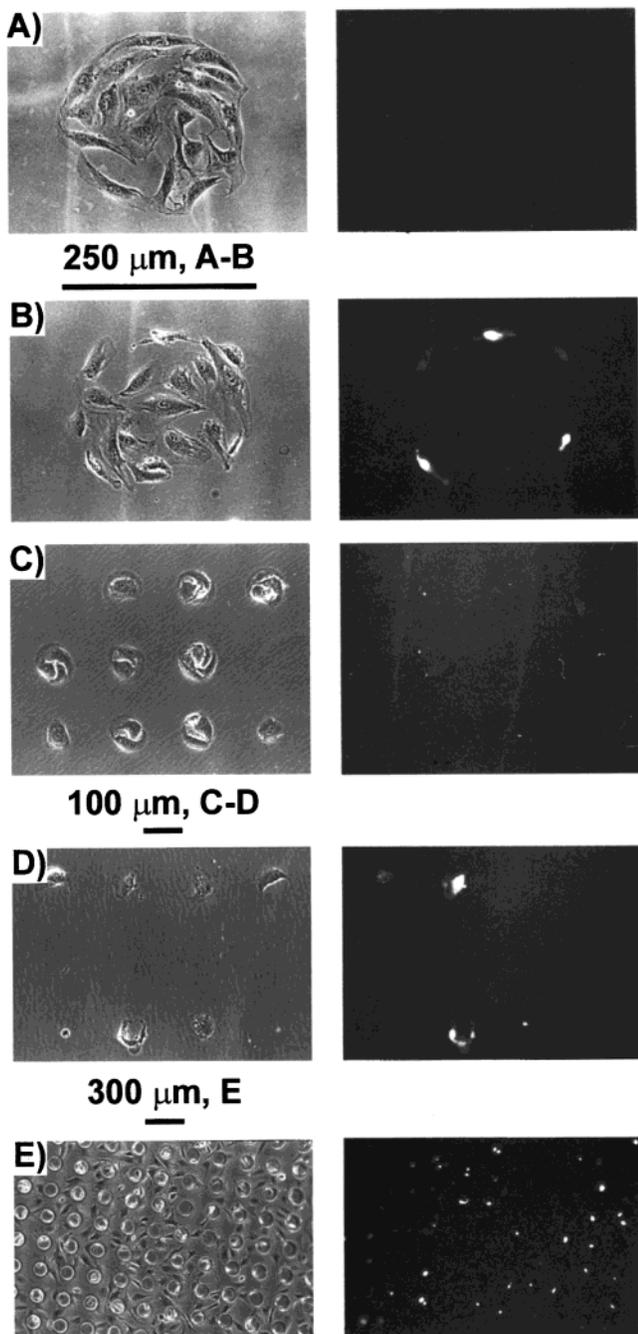
Figure 8. The elastomeric films must be coated with BSA to avoid damage to the membranes of cells during the lift-off step. The diameters of the features are 250 μm in A,B and 100 μm in C–E. The micrographs on the left side were obtained with phase-contrast microscopy, and the micrographs on the right side were obtained with fluorescence microscopy. (A) and (C) BCE cells were patterned on the substrate through a membrane that was coated with BSA using the procedure described in Figure 4. The cells were incubated with propidium iodide after the membrane was removed. The corresponding fluorescence micrograph shows that no cells internalized the fluorescent dye; this observation indicates that their membranes were not damaged. (B) and (D) Both membrane and substrate were coated with FN, using the procedure described in Figure 3, and cells adhered to both. Removal of the membrane in such experiments resulted in a poorly defined pattern of cells. Many of the cells that still adhered appeared to be damaged in the corresponding fluorescent micrograph. (E) The surface of the membrane that was used in (B) was covered by attached cells (Figure 6 A); many cells also adhered to the walls of the holes. A fluorescence micrograph of the membrane revealed that many of the cells that attached in the holes presented damaged membranes.

peeling away the BSA-coated membrane from the substrate; a small number of holes may have been poorly coated with BSA (hence adhesive) because of incomplete removal of air from the holes during the exposure to vacuum.

Cells that were simultaneously attached to the substrate and to the membrane were torn when the membrane was peeled away. The percentage of cells that incorporated PI was typically 20% when cells were patterned into holes with 250 μm diameters using membranes that were coated with FN; only the cells at the edges of the holes were damaged, and the cells in the center of the holes remained intact (Figure 8B). Circular features with a diameter of 100 μm were small enough that nearly every cell was in contact with the walls of the holes in the membrane; removing membranes of this size left few, and mostly damaged, cells on the substrate (Figure 8D, >50% of the cells incorporated PI). The cells that were attached to the walls of the holes of the removed membranes were also damaged (Figure 8E).

Spreading of Cells after Lift-Off. We used MEMPAT to study cell spreading by patterning FN or gelatin onto the substrates through BSA-coated membranes. Cells that were exposed to the assembly of membrane and substrate adhered selectively to the surface of the substrate that was exposed through the holes of the membrane. We allowed the cells to spread onto such substrates for 6–20 h until they covered the entire area exposed by the hole of the membrane. We then removed the membrane from the substrate in the presence of a solution of gelatin (free of BSA) and incubated it for 20 min. This procedure coated the areas of the substrates that had previously been covered by the membrane with a layer of adhesive protein. After replacing the solution of gelatin with culture medium, the cells were incubated for an additional 8 h. During that interval, the cells spread onto the rest of the surface of the substrate and covered it essentially in its entirety. If the experiments were allowed to proceed for an additional 24 h, a large fraction of the cells would have divided and covered the surface with a layer of cells more densely packed than that shown in Figure 9 (and eventually with a confluent monolayer).

Conclusions

MEMPAT Is a New Approach To Patterning the Attachment and Spreading of Cells on Surfaces. MEMPAT readily uses membranes that have regular features such as circles and squares. The ability to use other features (for example, rectangles or slots) depends on the stability of these features as the membrane is being manipulated. The membranes can be fabricated conveniently using rapid prototyping[16] provided the features have dimensions >20 μm.

MEMPAT makes cell patterning more accessible to biological laboratories than existing techniques. It is well-suited for patterning anchorage-dependent cells. A key advantage of MEMPAT is that it is applicable to a broad range of substrates; the only requirement of the technique is that the material should adsorb adhesion promoting proteins (FN, gelatin, vitronectin, collagen, laminin) spontaneously from solution, and that it be able to make conformal contact with the PDMS membrane. Many materials, including those used routinely in cell culture, satisfy those requirements. The membranes are reusable for at least a limited number of times (we have used one set of three membranes, three times each).

MEMPAT allows the patterning of cells within physical constraints that can be removed to allow cells to spread.
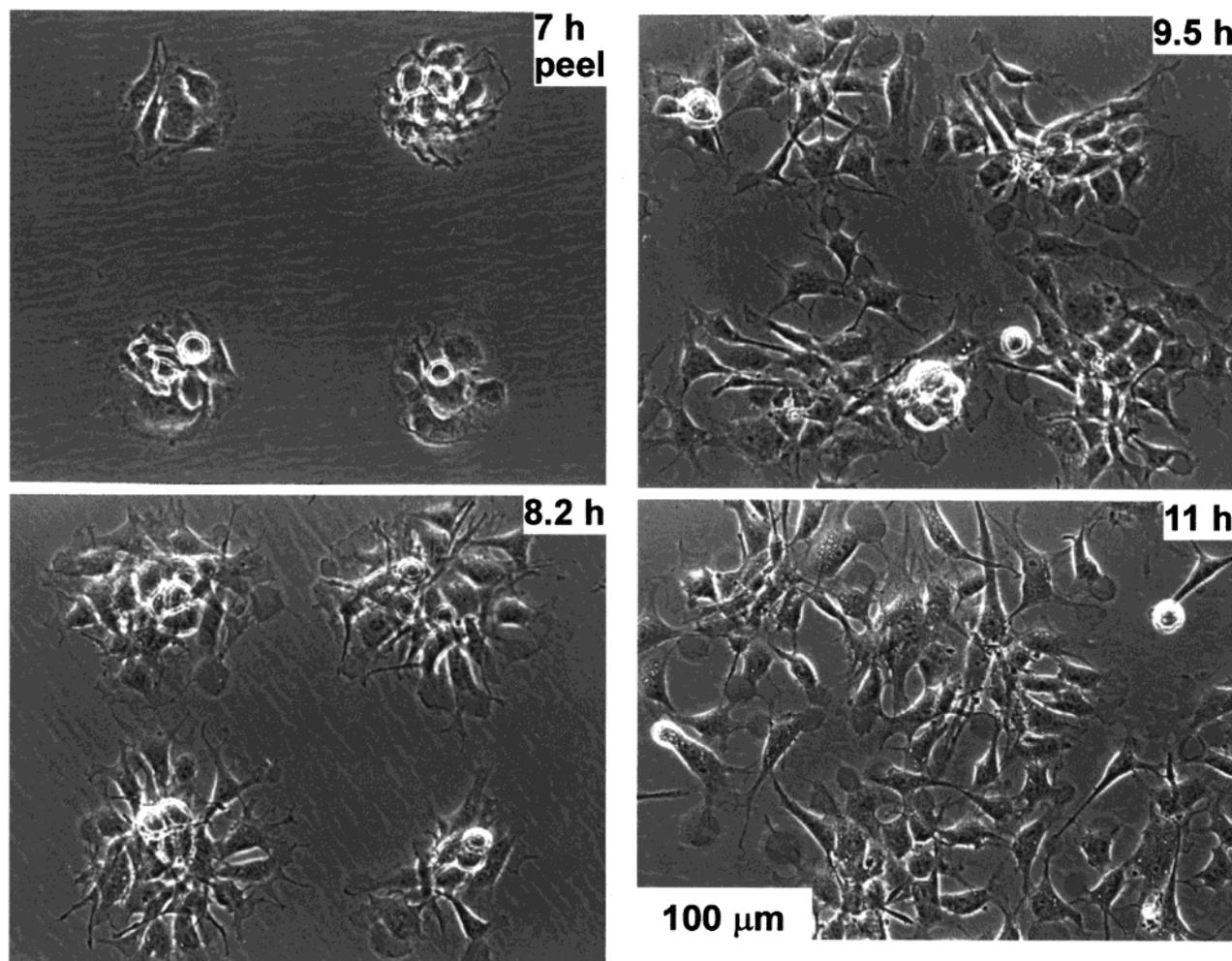
Figure 9. Demonstration that MEMPAT allows the study of cell spreading. BCE cells were patterned on Petri dishes using a BSA-coated membrane following the procedure described in Figure 4. After 7 h in culture, the substrates were rinsed with PBS and the membrane was removed. The four different samples were exposed to a solution of gelatin for 15 min following the procedure described in the text (Figure 4) and in the Experimental Section. The samples were incubated at 37 °C and at each indicated time from the beginning of the experiment, one of the samples was fixed and stained. Each image displays an area that is representative of the entire sample, but the areas are not the same.

In MEMPAT, cells are grown within "microwells" defined by the holes of the membranes and the surface of the substrate. We can control the surface chemistry of the walls of the membranes and the surface of the substrate in a way that causes cells to attach and spread on the substrate, but not attach to the membrane. The microwells can then be removed (or "disassembled") to allow cells to spread onto the rest of the surface.

New Directions in Cell Biology That Might Come from MEMPAT. MEMPAT can be used to pattern cell attachment using a physical constraint—the membrane. The ability to release the constrained cells by removing the membrane, and to observe their subsequent spreading, can be exploited in several areas of cell biology. The process of cell spreading and migration has, to date, been studied without controlling the shape and size of cells before allowing them to spread and migrate.[42] The shape and size of cells determines their passage through the cell cycle.[1,43] It is therefore possible that the size and shape of cells may also affect their ability to spread and migrate onto a homogeneous surface.

MEMPAT should also enable sophisticated studies of cell attachment on surfaces that present multiple types of proteins. In vivo, cells encounter many types of gradients or areas where the characteristics of the adhesive matrix change abruptly. MEMPAT could be used to study how cells spread onto a layer of protein after adhering to an island of a different protein.

Experimental Section

Materials. SU-8 50 photoresist was supplied by Microlithography Chemical Corp.(Newton, MA). We used rigid chrome masks (Advanced Reproductions, North Andover, MA) or transparencies as the photomasks in the photolithograpic step. Poly(dimethylsiloxane) (PDMS; Sylgard 184) was obtained from Dow Corning (Midland, MI). Bacteriological and tissue culture grade Petri dishes were purchased from Falcon. No. 2 glass slides from Corning Inc. (Corning, NY) were used as received. Silicon wafers ⟨110⟩ were obtained from Silicon Sense Inc. (Nashua, NH), and were also used as received. Phosphate-buffered saline packets were purchased from Sigma and diluted to the desired concentration (150 mM, pH = 7.4) with distilled water. Dulbecco's modified eagle medium (DMEM), BSA (fraction V), and fibronectin were purchased from Gibco (Life Technologies, Rockville, MD); we added 5 μM HEPES (JRH Biosciences, Lenexa, KS) to the medium. Sodium dodecyl sulfate (SDS) was purchased from Bio Rad (Hercules, CA). Gelatin was purchased from DIFCO

(42) Palecek, S. P.; Loftus, J. C.; Ginsberg, M. H.; Lauffenburger, D. A.; Horwitz, A. F. *Nature* **1997**, *385*, 537–540.

(43) Huang, S.; Chen, C. S.; Ingber, D. E. *Mol. Biol. Cell* **1998**, *9*, 3179–93.

Laboratories (Detroit, MI). Paraformaldehyde was purchased from Electron Microscopy Sciences (Ft. Washington, PA).

Substrates. We patterned cells on the surfaces of Petri dishes, PDMS, glass slides, silicon (⟨110⟩, native oxide). Unless specified otherwise, we always use Petri dishes as the substrates.

Fabrication of Patterned Photoresist Structures and Membranes.[40] Arrays of cylindrical posts of photoresist were fabricated on silicon wafers using standard photolithographic techniques and rigid chrome masks. The arrays of square features were fabricated using transparencies as photomasks. We used standard published procedures[40] to fabricate features that were 50 μm high.

Fabrication of Elastomeric Membranes. The membranes were fabricated using the procedure described by Jackman et al.[40] The PDMS prepolymer (mixed in a 10:1 ratio with a cross-linking catalyst) was spin-coated on the bas-relief of patterned photoresist using parameters known to produce a film that was thinner than the height of the features of photoresist. For features that were 50 μm tall, we spin-coated PDMS prepolymer at 3000 rpm for 60 s to generate a film that was ca. 45 μm thick. The PDMS films were cured for 2 h at 60 °C. A thicker layer of PDMS prepolymer was added to the edges of the membranes in dropwise fashion; after curing, this layer of PDMS provided a frame that would support the substrates; we typically used pieces that were 2 × 2 cm. The films were kept at 60 °C overnight. Prior to use in cell culture, we removed low molecular weight polymer from the membranes by soaking them in dichloromethane for 12 h. The membranes were then soaked in ethanol for 1 h and dried in an oven at 60 °C for 12 h. The membranes were removed from their supports using tweezers and they were then cut to the desired sizes along the edges of the support. The membranes generally come into conformal contact with the substrates. In cases when the membranes were not flat on the surface and adhered to themselves, we placed a drop of ethanol on them to facilitate the formation of a flat seal. The ethanol wets the surface of the membrane preferentially and it allows it to become flat; evaporation of the ethanol leaves the membranes flat on the substrates. The membranes were ready for use after evaporation of the ethanol.

Procedure Used To Wash the Membrane After Use in Cell Culture. The membranes were kept in buffered SDS (10 mg/mL, PBS at pH = 7.4) for 30 min at room temperature and 30 min at 90 °C, followed by extensive rinsing with deionized water and ethanol. The membranes were then extracted with dichloromethane for 12 h and dried at 60 °C for 12 h. These membranes (like other microscopic structures made of PDMS) can also be sterilized by autoclaving (20 min at 121 °C, 115 kPa).

Modification of Surfaces. *(a) Coating the Membrane with BSA*. In a laminar flow hood, the membranes were placed on the surface of a sterile Petri dish with a few drops of ethanol. The liquid sterilized the membranes by killing bacteria. Drops of a buffered solution of BSA (1% w/v, in PBS or DMEM at pH = 7.4) were placed on the membrane to cover the holes. Since the liquid did not fill the hydrophobic pores, we applied (ca. 30 s) and released vacuum (ca. 500 mTorr) twice to extract the air trapped in the pores (Figure 2) and allowed BSA to adsorb to the surfaces for 15 min. The substrates were then rinsed three times with PBS; the membranes were peeled from the support in the presence of PBS, and transferred to a clean Petri dish covered with PBS to help seal the membrane onto the dish.

(b) Patterning Proteins on Substrates Using MEMPAT. Drops of buffered fibronectin (50 μg/mL, PBS with pH = 7.4) or gelatin (1.5% w/v, PBS with pH = 7.4) solutions were placed on a membrane in conformal contact with a substrate. We applied (ca. 30 s) and released vacuum twice to extract the air trapped in the pores (Figure 2) and allowed the protein to adsorb to the surfaces for 1 h (FN) or 15 min (gelatin). The assembly of the membrane and substrate was then rinsed with buffer 3 times. The membrane was removed from the surface with a pair of tweezers, in the presence of culture media that contained 1% (w/v) BSA. After 15 min, fresh media was introduced into the dish, followed by a suspension of cells.

Immunofluorescent Staining of Adsorbed FN. The substrates coated with FN were exposed to 4% (v/v) PFA in PBS buffer (pH = 7.4) for 20 min, and then immersed in a solution of rabbit anti-human fibronectin IgG (Sigma, 5 μg/mL) for 1 h.

The substrates were rinsed twice with PBS containing 0.1% (w/v) BSA and 0.1% (v/v) Triton X-100, and placed in contact with 100 μL of Texas Red-labeled goat anti-rabbit IgG (Amersham Life Sciences, 50 μg/mL) for 1 h; the samples were then rinsed, and sealed onto microscope slides with Fluoromount-G (Southern Biotechnology, Inc.).

Cell Culture. *(a) Growth and Attachment*. Bovine adrenal capillary endothelial (BCE) cells were cultured under 10% CO_2 on cell culture Petri dishes (Falcon) coated with gelatin in DMEM containing 10% calf serum, 2 mM glutamine, 100 μg/mL streptomycin, 100 μg/mL penicillin, and 1 ng/mL basic fibroblast growth factor (bFGF).[2] Prior to incubation with the patterned substrates prepared using MEMPAT, cells were dissociated from culture plates with trypsin−EDTA and washed in DMEM containing 1% BSA (BSA/DMEM). The suspension of cells (typically 25000 cells/mL, 2 mL total volume) was placed on the substrates in chemically defined medium (10 μg/mL high-density lipoprotein, 5 μg/mL transferrin, 5 ng/mL basic fibroblast growth factor in 1% BSA/DMEM) and incubated in 10% CO_2 at 37 °C.[2] A typical incubation time was 24 h.

(b) Fixing and Staining Cells. Substrates that contained cells were fixed with PFA for 20 min and washed with PBS. The substrates were then washed with methanol for 1 min, and stained with Coomassie Blue (5 mg/mL in 40% v/v methanol, 10% v/v acetic acid, and 50% v/v water) for 30 s; they were then rinsed with distilled water and dried in air.

Procedures Used to Study Cell Spreading. Cells were allowed to attach to patterns of gelatin or FN defined by the holes of the BSA-coated membranes**.** After 7−24 h, the assembly defined by the membrane, the substrate, and the attached cells was rinsed with PBS buffer three times to remove BSA from the solution and it was then immersed in a PBS solution of gelatin (1.5% w/v). The membrane was peeled gently from the surface with a pair of tweezers and the substrates were incubated for 15 min to adsorb gelatin on the areas of the surface that were protected by the membrane. The substrates were then rinsed once with culture medium (DMEM) before being placed in the incubator for ca. 4 h, to allow the cells to spread onto the previously protected areas of the substrates.

Characterization of Damage to Cells. Membranes were gently removed from substrates that presented attached cells. The attached cells were incubated with a solution of propidium iodide in culture medium (10 μg/mL) for 15 min. The cells were imaged with a fluorescence microscope immediately after rinsing the samples twice with culture medium at 37 °C. The intensity of the fluorescence of propidium iodide decreased as the dye diffused out of the cells over the course of 2 h; this diffusion into the medium also decreased the contrast obtained in the micrographs.

Microscopy. (a) *Phase Contrast and Fluorescence Microscopy*. These studies were performed with a Nikon Axiophot equipped with a 35 mm camera. The developed negatives or slides were scanned into a digital format with a Nikon LS-400 slide scanner. Images were processed only by performing operations uniformly on the entire image; we typically converted the color images to black and white and enhanced the contrast to ensure that the fine features of the cell structure would appear in the version of the figure printed in the journal.

(b) SEM Micrographs. These were obtained on a JEOL JSM-6400 scanning electron microscope operating at 15 keV.