Microfabricated multilayer parylene-C stencils for the generation of patterned dynamic co-cultures

Satoshi Jinno,[1,2,5]* Hannes-Christian Moeller,[1,3*] Chia-Ling Chen,[4] Bimal Rajalingam,[1,5] Bong Geun Chung,[1,5] Mehmet R. Dokmeci,[4] Ali Khademhosseini[1,5]

[1]*Department of Medicine, Center for Biomedical Engineering, Brigham and Women's Hospital, Harvard Medical School, Boston, Massachusetts 02115*

[2]*Department of Periodontology, Aichi Gakuin University School of Dentistry, Nagoya, Japan*

[3]*École Supérieure de Biotechnologie de Strasbourg, F-67412, Illkirch-Cedex, France*

[4]*Electrical and Computer Engineering Department, Center for High Rate Nanomanufacturing, Northeastern University, Boston, Massachusetts 02115*

[5]*Harvard-MIT Division of Health Sciences and Technology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139*

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Abstract: Co-culturing different cell types can be useful to engineer a more *in vivo*-like microenvironment for cells in culture. Recent approaches to generating cellular co-cultures have used microfabrication technologies to regulate the degree of cell–cell contact between different cell types. However, these approaches are often limited to the co-culture of only two cell types in static cultures. The dynamic aspect of cell–cell interaction, however, is a key regulator of many biological processes such as early development, stem cell differentiation, and tissue regeneration. In this study, we describe a micropatterning technique based on microfabricated multilayer parylene-C stencils and demonstrate the potential of parylene-C technology for co-patterning of proteins and cells with the ability to generate a series of at least five temporally controlled patterned co-cultures. We generated dynamic co-cultures of murine embryonic stem cells in culture with various secondary cell types that could be sequentially introduced and removed from the co-cultures. Our studies suggested that dynamic co-cultures generated by using parylene-C stencils may be applicable in studies investigating cellular interactions in controlled microenvironments such as studies of ES cell differentiation, wound healing and development. © 2008 Wiley Periodicals, Inc. J Biomed Mater Res 86A: 278–288, 2008

Key words: cellular microenvironment; dynamic co-culture; parylene-C stencils; cell patterning

INTRODUCTION

The signals in the cellular microenvironment comprise of physicochemical factors, mechanical signals as well as cell–cell, cell-soluble factor, and cell–extracellular matrix (ECM) interactions.[1] Specifically, direct or indirect cell–cell interactions have been implicated in regulating a variety of cellular responses. Adjacent cells in contact are able to communicate with each other via cell adhesion molecules, surface receptors, and even direct cytoplasmic connections. Cell–cell interactions can be stable, such as interactions in epithelial cell sheets, where cells are connected via cell–cell junctions, or transient, such as the interactions between cells of the immune system. Cellular cross-talk is also mediated via soluble cues provided through autocrine or paracrine signaling. In addition, the ECM is also a key regulator of cell behavior. The ECM consists of a complex mixture of secreted structural and functional macromolecules and acts as an important mediator of cell signaling.[2] The ECM is a reservoir for soluble signaling molecules, and its biological, physicochemical, and mechanical properties have been shown to regulate cell behavior.[3–5] Collagen, fibronectin (FN), laminin, and hyaluronic acid (HA) are important ECM components and have been widely used for tissue engineering and *in vitro* cell culture.

*These authors contributed equally to this work.

Correspondence to: A. Khademhosseini, 65 Landsdowne Street, Room 252, Cambridge, MA 02139, USA; e-mail: alik@mit.edu

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The complex and dynamic interplay of microenvironmental stimuli mediated by the ECM and cellular cross-talk regulates cell fate decisions, proliferation, self-renewal, and migration.[6,7] The temporal aspects of these stimuli and their cellular responses are of key importance in biological processes related to stem cell differentiation, morphogenesis, and wound repair.[8–11] Thus, the ability to control the cellular microenvironment in a temporally controlled manner has significant potential in the study of a number of biological processes and it could be useful in regulating cell behavior for applications in regenerative medicine.[12,13]

The *in vitro* deposition of ECM and the subsequent assembly of cells with control over cell position[14] and spatial organization[15] are of great importance in tissue engineering.[1] Previously, patterned co-cultures of two or more cell types have been generated using approaches such as photolithography,[16–19] layer-by-layer deposition of cell-adhesive materials,[12,20] elastomeric membranes,[21–24] and microfluidics.[25] Despite their usefulness in controlling the cellular interactions these approaches cannot be used to control the dynamic cell–cell contact.

To control cell–cell contact dynamically, Hui and Bhatia have developed a comb-like microchip that was used to control the interactions between hepatocytes and stromal cells.[13] As an alternative to this approach our group developed a simple patterning technique that could be applied for dynamic co-cultures of a variety of cells. In this approach we used surface engineering to reversibly change the adhesiveness of parylene-C stencils to enable dynamic seeding of support cells around a primary cell type. The parylene-C stencils could then be removed to expose the underlying substrate and allow seeding of a subsequent cell type on the exposed regions.

The previous approach used parylene-C stencils which are biocompatible,[26] mechanically robust,[27] and suitable. A major constraint of our previous approach, however, was that it could not be applied for the sequential co-culture of more than two cell types.

In this study, we develop multilayer parylene-C stencils and investigate their potential for patterning ECM proteins and cells. Using a multilayer parylene-C stencil, we describe a novel, rapid, and convenient method for the generation of dynamic co-cultures of at least five different cell types, in which cell–cell, cell-soluble factors and cell–ECM interactions can be controlled in a spatiotemporal manner.

MATERIALS AND METHODS

Preparation of multi-layer parylene-C stencils

Three-inch silicon wafers were cleaned with piranha solution (1:1 H_2SO_4:H_2O_2) for 10 min, rinsed in deionized water, nitrogen dried, and baked for 10 min at 150°C. The wafers were then coated with hexamethyldisilazane (HMDS) to facilitate eventual removal of the finished parylene-C stencil. A thin (5 μm or 10 μm) film of parylene-C was first deposited on a silicon wafer using a PDS 2010 Labcoater 2 Parylene-C Deposition System (Specialty Coating Systems, Indianapolis, IN). For successful removal of individual stencil layers, an anti-stiction layer (detergent, micro 90, International Products Corporation, Burlington, NJ) was applied via spin coating between each of the parylene-C layers. The detailed fabrication process of stencils with microwell pattern is illustrated in Figure 1. A 200-nm thick aluminum layer was deposited and patterned as a hard mask. Microwells were created on the parylene stencil utilizing low temperature (5°C) dry etching in an inductively coupled plasma reactor (Plasmatherm 790). After etching, the aluminum hard mask was removed by using PAN etchant at 50°C for 2 min. Using this technique, we fabricated three designs (with layer thicknesses of, from top to bottom, 5–5–5 μm, 5–5–10 μm, and 10–10–10 μm) of peelable multilayer parylene-C stencils. In all stencils, the microwell diameter was 200 μm.

Scanning electron microscopy imaging of parylene-C stencils

Stencils were analyzed via scanning electron microscopy (SEM) using a Zeiss Supra 25 (Carl Zeiss Microscopy, Jena, Germany). After fabrication, the stencils were cut in half. Cross-sectional views were then obtained using SEM to characterize the profiles and thicknesses of the stencil layers.

Contact angle measurements of parylene-C stencils

Contact angles were measured for static drops of water on four different substrates-poly(dimethylsiloxane) (PDMS), parylene-C, parylene-C coated with detergent, and plasma-treated parylene-C using a contact angle measurement system (Phoenix 300 plus, SEO Surface Electro Optics, Korea). Measurements were obtained after dispensing de-ionized water drops onto each substrate using a micropipette (Ted Pella, Redding, CA). Each data point represents an average of at least 10 independent measurements.

Patterning of multiple proteins

Multilayer parylene-C stencils were reversibly sealed on a PDMS surface and the individual layers were subsequently patterned with BSA coupled to different fluorophores. First, both the top stencil layer and the PDMS substrate exposed through the stencil microwells were coated for 30 min with 100 μg/mL fluorescein isothiocyanate (FITC) coupled to BSA. The top stencil was then gently peeled off using tweezers to yield an FITC-BSA protein pattern only inside the 200-μm microwells. Subsequently, the next stencil layer was patterned with 50 μg/mL Texas Red-BSA (TR-BSA) for 30 min to yield a co-pattern with the FITC-BSA. The middle layer was then peeled off, and

the underlying (bottom) stencil layer was coated for 30 min with 50 μg/mL 6-((7-Amino-4-methylcoumarin-3-acetyl)amino)hexanoic acid (AMCA) coupled to BSA, yielding another protein co-pattern. Finally, after removal of the bottom stencil, the PDMS surface was patterned with 50 μg/mL TR-BSA for 30 min. Images of parylene-C stencils co-patterned with fluorescent BSA were taken at 4× and 10× magnifications using an inverted fluorescent microscope (Nikon Eclipse TE2000-U).

FITC-BSA and TR-BSA were purchased from Sigma–Aldrich (St. Louis, MO), except AMCA-BSA. To synthesize AMCA-BSA, BSA was conjugated with the blue fluorophore AMCA using the AnaTag AMCA-X microscale protein labeling kit, ANASpec (San Jose, CA).

Cell culture

Pluripotent murine embryonic stem (ES) cells (R1 strain),[28] were purchased from the Mount Sinai Hospital (Toronto, Canada). Murine epithelial ameloblast-lineage cells (ALCs)[29] were obtained from The Forsyth Institute (Boston, MA). All other cell lines were purchased from American Type Culture Collection (Manassas, VA). Cells were manipulated under tissue culture hoods and maintained in a humidified incubator at 37°C with a 5% CO_2 atmosphere. Tissue culture components were purchased from Gibco-Invitrogen (Carlsbad, CA), unless otherwise indicated. ES cells were maintained on gelatin treated dishes in knockout Dulbecco's modified eagle medium (DMEM) supplemented with 15% (v/v) ES qualified fetal bovine serum (FBS), 1% (v/v) nonessential amino acid solution MEM NEAA, 1 m*M* L-glutamine, 0.1 m*M* 2-mercaptoethanol, and 10^3 U/mL mouse leukemia inhibitory factor (LIF), ESGRO® (Chemikon, Eugene, OR). ES cells were kept undifferentiated by daily media changes and by passaging every 2 days at a subculture ratio of 1:4. All other cell types were fed every other day and passaged when 90% confluency was reached. ALCs were maintained in a medium comprised of Spinner modified DMEM containing L-glutamine, supplemented with 10 ng/mL rhEGF, 0.2 m*M* calcium, 1% (v/v) penicillin–streptomycin, and 10% (v/v) heat-inactivated FBS. NIH-3T3 mouse fibroblasts were maintained in DMEM medium with 10% (v/v) FBS. Normal human umbilical vein endothelial cells (HUVECs) were maintained in endothelial cell basal medium from Clonetics EGM-2 Simple Quads (Lonza, Walkersville, MD). HL-1 murine cardiomyocytes[30] were maintained in Claycomb media (SAFC Biosciences, Lenexa, KS) with 1% norepinephrine, 1% (v/v) L-glutamine, 1% (v/v) penicillin–streptomycin and 10% (v/v) FBS.

Cell adhesion on parylene-C surfaces

NIH-3T3 cells were seeded onto detergent-treated and untreated parylene-C stencils at a density of 1000 cells/mm^2 and incubated for 4 h. After incubation, non-adherent cells were washed away by immersing the stencils in phosphate buffered saline (PBS). To facilitate visualization of the adherent cells on the parylene-C stencil, cells were previously labeled using 40-6-diamidino-2-phenylindole (DAPI) for 5 min. Several randomly selected fields were imaged using an inverted fluorescent microscope Nikon Eclipse TE 2000U (Nikon, Tokyo, Japan), and cells were counted. Cell adhesion on detergent-treated and untreated parylene-C stenci\ls was compared. Statistical analysis was performed using two-tailed multiple *t*-test with Bonferroni correction following two-sided analysis of variances (ANOVA), with $p < 0.05$ considered statistically significant.

Cell staining

To visualize various cell types in patterned co-cultures, cells were stained with fluorescently labeled dyes and tracked in culture. The stains used were carboxyfluorescein diacetate succinimidyl ester (CSFE) (Invitrogen, Eugene, OR) for the mES cells and PKH26 (Sigma–Aldrich, St. Louis, MO) for other cell types. Staining with CFSE was performed by incubating ES cells at a concentration of 1×10^7 cells/mL in a 10-μg/mL CFSE solution for 10 min at 25°C. To stain with PKH26, cells (1×10^7 cells/mL) were suspended in a 4×10^{-6} *M* PKH26 staining solution. The cells were then incubated at 25°C for 5 min. Both staining reactions were quenched via the addition of an equal volume of DMEM supplemented with 10 % (v/v) FBS, followed by washing. All cell types that were co-cultured with CFSE-labeled ES cells were stained red using PKH26. For visualization, however, each cell type was later assigned a specific color using Adobe Photoshop software (Adobe Systems, Salinas, CA).

Generation of dynamic co-cultures

Dynamic co-cultures were generated as outlined in Figure 2. PDMS substrates were fabricated by curing a silicone elastomer solution mixed in a 10:1 ratio with curing agent Sylgard 184, (Dow Corning, Midland, MI) inside a Petri dish for 2 h. The PDMS substrates were then coated with a 20 μg/mL FN solution for 1 h. Multilayer parylene-C stencils were first incubated with HA (5μg/mL) for 1 h. After incubation, stencils were washed and reversibly sealed on FN-treated PDMS substrates inside Petri dishes. ES cells (~5000 cells/mm^2) were then seeded onto the parylene-C stencils and incubated for 6 h at 37°C. Cells selectively adhered to the FN-coated PDMS surface through the 200 μm holes in the stencil (because the top parylene-C layer was cell-repellent due to HA coating). The top surface of the stencil was next coated for 10 min with a 500 μg/mL collagen solution. HUVECs were then seeded onto the stencil surface (~5000 cells/mm^2) and incubated for 4 h. After incubation, the HUVECs were removed by peeling off the top layer of the stencil. Another collagen coating was applied for 10 min. Next, ALCs were seeded (~5000 cells/mm^2) and again incubated with the ES cells for 4 h. The ALCs were then removed by peeling away the second parylene-C layer. Before seeding the fourth cell type, the last remaining parylene-C layer was coated with collagen. NIH-3T3 cells were then seeded at a density of ~5000 cells/mm^2 and incubated for 4 h with the ES cells before the bottom layer of the parylene-C stencil was removed from the PDMS surface. Finally, the PDMS surface was coated with FN at a concentration of 20 μg/mL for 10 min, and HL-1 cells were seeded (~5000 cells/mm^2)

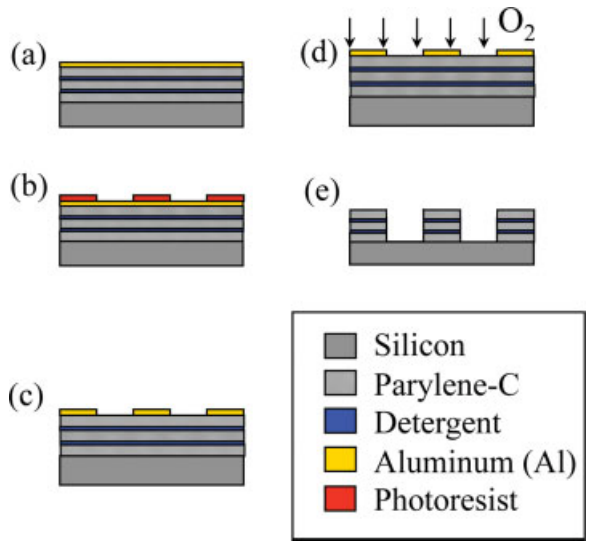

Figure 1. Fabrication process for multilayer parylene-C stencils with a microwell pattern. A thin layer of detergent was applied to each parylene-C layer to generate a peelable multilayer stencil. An aluminum hard mask was deposited on top of the stencil (a). To create the microwell pattern, a thin layer of photoresist was spun and patterned on the substrate (b) and subsequently transferred to the aluminum hard mask by submersing the wafer in an aluminum etchant at 50°C for 1 min (c). Microwell patterns were then generated using low temperature (5°C) dry etching with O_2 plasma (d). After the dry etch process, the aluminum hard mask was removed (e). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

as the fifth cell type. The ES cells were co-cultured for another 4 h with the HL-1 cells.

Analysis of the number of ES cells retained

We analyzed the number of ES cells that were retained inside wells after peeling off the individual films. Parylene-C stencils of different film thicknesses (5–5–5, 10–5–5, and 10–10–10 μm) were compared for their ability to retain ES cells during dynamic co-culturing. For different stencils, the number of ES cells inside randomly selected microwells was counted after each step of the process described above. The obtained mean values were evaluated, and statistical analysis was performed using a two-tailed multiple *t*-test with Bonferroni correction, followed by a two sided analysis of variances (ANOVA), with $p < 0.05$ considered statistically significant.

RESULTS AND DISCUSSION

We have previously reported the generation of static and dynamic patterned co-cultures using microfabricated parylene-C stencils.[23,24] To generate dynamic co-cultures, we used layer-by-layer surface modifications combined with parylene-C stencils. The previous system, however, could not be applied for the sequential co-culture of more than two cell types. To overcome this limitation and to develop a method for sequential co-cultures, we designed and fabricated multilayer parylene-C stencils by spin-coating a thin layer of detergent between the parylene-C layers. Our stencils consisted of three individ-

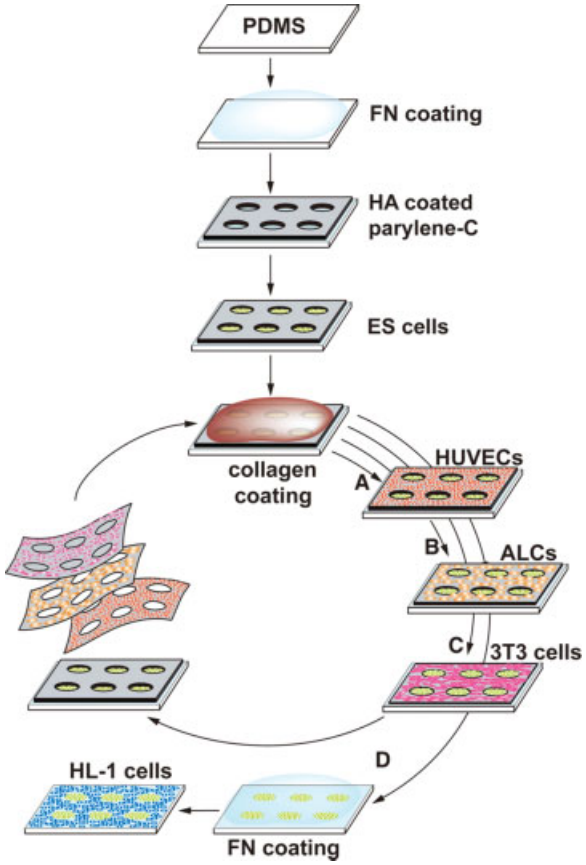

Figure 2. A schematic representation of the dynamic co-culture system. The PDMS substrate is coated with a gelatin/fibronectin solution whereas the three-layered parylene-C stencil is incubated with HA to render it nonadhesive. After treatment, the stencil is reversibly sealed onto a PDMS substrate. ES cells are seeded onto the stencil and dock inside the microwells, where they adhere to the exposed gelatin/fibronectin-coated PDMS surface. The HA-coated top layer of the parylene-C stencil is resistant to cell adhesion, and cells that are settled in this region can easily be removed by pipetting a gentle stream of media over the stencil. After patterning the ES cells, the HA-coated parylene-C stencil is treated with a collagen solution, rendering it cell-adhesive. HUVECs are seeded onto the stencil and adhere to the top layer (A). The two cell types are co-cultured until the top stencil is peeled away, removing the adherent HUVECs with it. The middle stencil layer underneath is coated with collagen before cells of the third cell type (ALC) are seeded onto the stencil to perform a second co-culture (B). The peeling and protein coating steps are repeated, and NIH-3T3 and HL-1 cells are seeded as the fourth and fifth cell types, respectively (C, D). The duration of the co-cultures and the series of cell types can be varied depending on the specific application. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

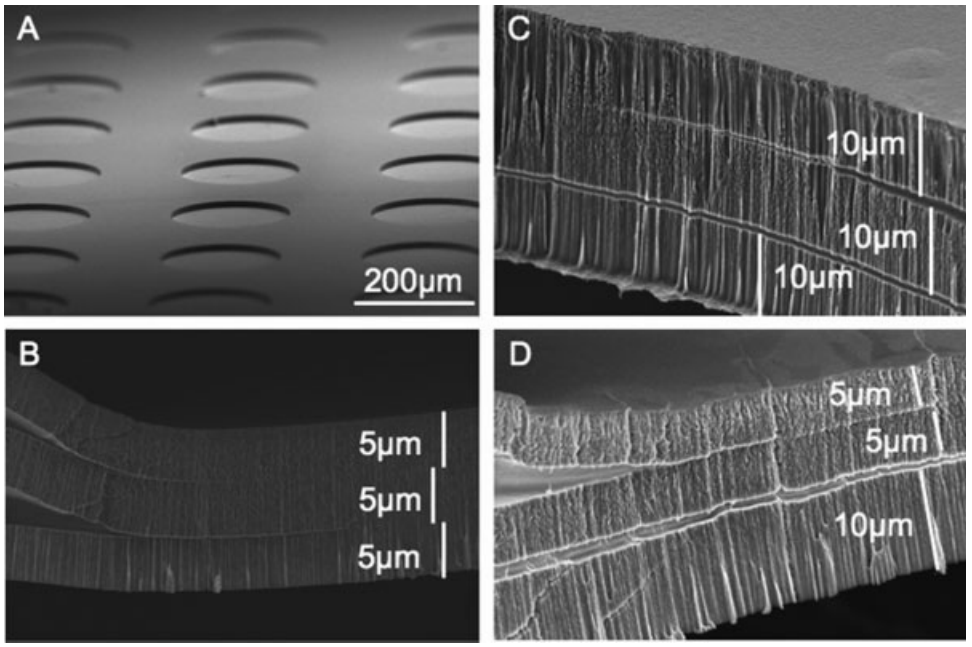

Figure 3. A scanning electron microscope (SEM) image of parylene-C stencils. An oblique view on the top of the stencil displays a pattern of microwells with 200 μm diameter with almost vertical side walls (A). The three-layered parylene-C stencils were engineered with three combinations of layer thicknesses. Cross-sectional images of the stencils were taken at higher magnification to depict the individual layers. The images show three-layered parylene-C stencils with individual film thicknesses of (from top to bottom) 5–5–5 μm (B), 10–10–10 μm (C), and 5–5–10 μm (D).

ually peelable layers of parylene-C of defined thicknesses (5 or 10 μm). We characterized the multilayer stencils using SEM and then analyzed cell adhesion on detergent-treated parylene-C surfaces to evaluate potential biological consequences of the fabrication method. We then developed a multi-step process for the generation of dynamic protein and cell co-patterns using our multilayer stencils. We analyzed the influence of the thicknesses of the individual layers on the number of cells retained inside microwells during co-culture. Changing the heights of the individual layers enabled us to optimize our system to retain cells within our pattern. Finally, we demonstrate that four sequential co-cultures (ES cells cultured with HUVECs, ALCs, NIH-3T3 cells, and HL-1 cells) can be achieved utilizing our dynamic co-culture system.

Characterization of the multi-layer parylene-C stencils

We fabricated multilayer stencils with three different layer thickness combinations. The amount of the parylene-C dimer used during the vapor deposition step dictates the amount of deposition, thereby allowing us to specify stencil layer thicknesses. SEM micrographs of the multilayer stencils used in our cell culture experiments are shown in Figure 3. In previous work, we had found that microwells with a 200-μm diameter were suitable for accommodating multiple cell types, and hence this diameter was chosen for our current study[24] [Fig. 3(A)]. The thickness combinations we fabricated were (from top layer to bottom) 5–5–5, 10–10–10, and 5–5–10 μm. Cross-sectional views show that the fabricated stencils are comprised of the three individual layers of specified thicknesses [Fig. 3(B–D)]. Utilizing a low temperature plasma etch enabled us to achieve vertical side walls of the stencil microwells. The images in Figure 3 further illustrate that the individual layers of parylene-C can be independently peeled from each other.

The surface properties of the parylene-C membranes determine various critical system parameters, such as protein adsorption, cell adhesion, and the bonding affinity between consecutive layers and thus the ease of peeling process.[31] To characterize the hydrophilicity of the parylene-C surfaces, we measured contact angles of stencils that were coated with detergent or treated with plasma (Fig. 4). The surfaces of untreated parylene-C stencils manifested a hydrophobicity similar to PDMS, which was used as a substrate in subsequent cell and protein patterning experiments. When the parylene-C membranes were treated with reactive oxygen plasma, however, their surfaces became more hydrophilic. The contact angle of untreated parylene-C stencils was ~9 times higher than that of plasma-treated parylene-C sten-

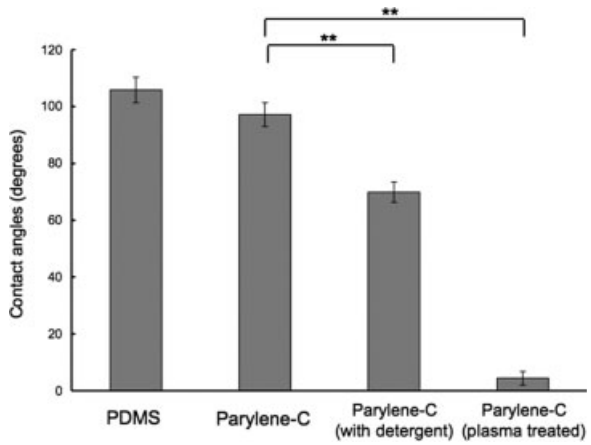

Figure 4. Contact angles for various materials and surface conditions of the parylene-C stencils. The surfaces of the untreated parylene-C stencils were hydrophobic, similar to the PDMS substrate. However, when parylene-C membranes were treated with either detergent or reactive oxygen plasma, their surfaces became more hydrophilic. The contact angle measured for parylene-C was significantly different from angles measured for detergent- and plasma-treated parylene-C (**$p < 0.01$).

cils. Interestingly, detergent-treated stencils were significantly more hydrophilic than untreated parylene-C stencils (Fig. 4).

Patterning of multiple proteins

We successfully patterned BSA labeled with various fluorophores using multilayer parylene-C stencils (Fig. 5). Initially, the entire surface of the parylene-C stencil was coated with FITC-BSA [Fig. 5(A)]. After the top layer of the parylene-C stencil was removed, a precise pattern of protein was retained inside the 200-μm microwells of the stencil [Fig. 5(B)]. With a concentration of 100 μg/mL FITC-BSA, the PDMS surface on the bottom of the microwell pattern was completely blocked and was therefore resistant to additional protein deposition. This enabled us to obtain co-patterns during subsequent coating steps without depositing TR-BSA or AMCA-BSA within the FITC-BSA patterned microwell bottoms [Fig. 5(C–E)]. Furthermore, the FITC-BSA protein pattern remained stable even after multiple protein co-patternings, and the integrity of the pattern was not affected by peeling away the stencil layers. These results demonstrate that proteins can be selectively patterned at microscale resolution using parylene-C stencils. Protein patterns can easily be varied by changing the geometry of the stencil. The technology further allows for the generation of dynamic co-patterns of multiple proteins. Selective patterning of proteins such as antibodies and enzymes, for example, has recently attracted much interest for the study of specific protein–protein interactions and the development of diagnostic kits, protein sensors, and protein chips.[32–35] Parylene-C technology might find application in this rapidly developing field of selective protein patterning.

Cell adhesion on parylene-C stencils

During the fabrication of the stencils, thin films of detergent were deposited between the individual stencil layers. The detergent films served to prevent adhesion between the layers. Despite this advantage, it was reasoned that exposing cells to detergent could adversely affect cell adhesion. To examine this possibility, we compared adhesion of NIH-3T3 cells on detergent-treated and untreated parylene-C stencils. Stencils were also treated with the same ECM components used for generation of multiple co-cultures (namely HA, collagen, and layer-by-layer deposition of collagen on HA). The results are depicted in Figure 6. Surface treatment of parylene-C stencils with these ECM components generally increased cell adhesion (as measured after 4 h), which is in accordance with observations documented in our previous work.[23] Our data show a switch from a cell-repellent HA-coated parylene-C surface to a cell-adhesive surface after layer-by-layer deposition of collagen onto HA. However, when detergent-treated parylene-C membranes were compared to untreated ones, our results showed that detergent treatment had an adverse effect on cell adhesion if no additional surface treatment was performed (Fig. 6). Cell adhesion on uncoated, detergent-treated parylene-C membranes was low. However, after surface treatment with any of the applied ECM components used in our experiments, cell adhesion was restored to the extent observed with untreated membranes (Fig. 6). We hypothesize that surface treatment with collagen and FN improved cell adhesion by forming a layer above the detergent film, and the presence of the biocompatible ECM molecules thus attenuated the observed effect of detergent treatment on cell adhesion.

Dynamic co-cultures on parylene-C stencils and stability of patterned cells

We next investigated dynamic cell co-culture using multilayer parylene-C stencils. The sequence of images in Figure 7 shows a dynamic co-culture involving five different cell types, achieved utilizing a 3-layer parylene-C stencil. Selective patterning of ES cells (green) was achieved by preferential docking of the cells inside the stencil microwells. Nonspecific cell adhesion onto the top layer of the stencil was

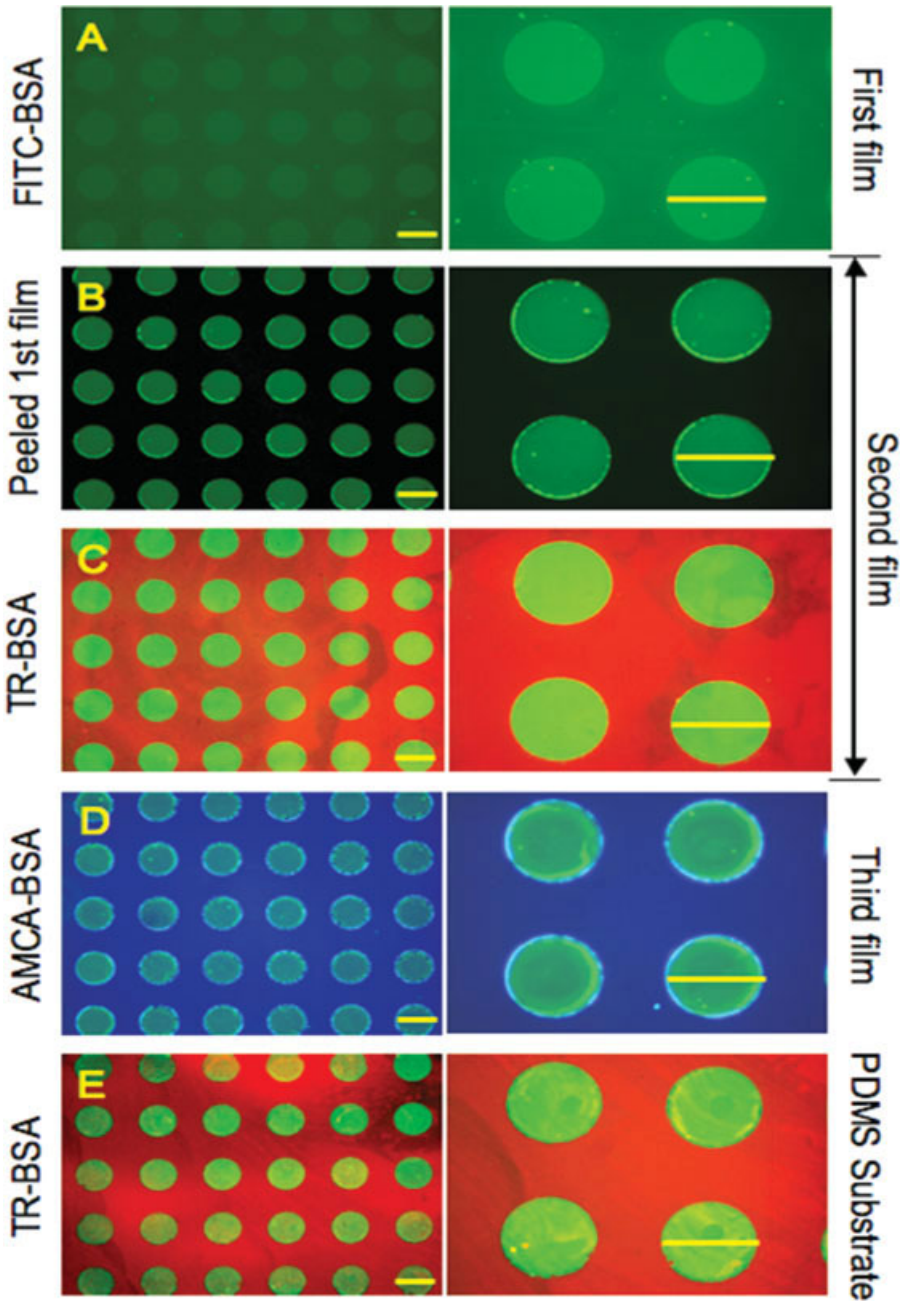

Figure 5. Patterning of multiple proteins using a multilayer parylene-C stencil. Parylene-C films were placed on PDMS and were patterned with FITC-BSA (A). Peeling off the top stencil layer yielded a pattern of FITC-BSA inside the microwells of the parylene-C stencil (B). A co-pattern was generated by patterning TR-BSA (C) onto the middle stencil layer. This layer was next peeled off (the pattern after peeling is shown only once in (B)), and the surface of the bottom parylene-C layer was coated with AMCA-BSA (D). Finally, the bottom stencil layer was peeled off, and the underlying PDMS substrate was patterned with TR-BSA (E). These images indicate that FITC-BSA protein could adsorb to the PDMS substrate inside the microwells and was retained even after multiple co-patterns (Scale bars = 200 μm). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

inhibited with an initial coating of HA. The cell-repellent properties of HA-coated surfaces have been previously described and used for cell patterning.[24,26,36] After ES cells were localized to the microwells, a layer-by-layer deposition approach using collagen was applied to restore cell-adhesive properties to the top stencil surface [Fig. 7(A–C)]. Surface switching of parylene-C from cell-repellent to cell-adhesive has been previously described and used for selective cell patterning.[12,23,24] Sequential switching of adhesive properties of the top layer of the parylene-C stencil enabled us to pattern a sequence of secondary cell types around (but not within) the ES cell-containing microwells: HUVECs [Fig. 7(D–F),

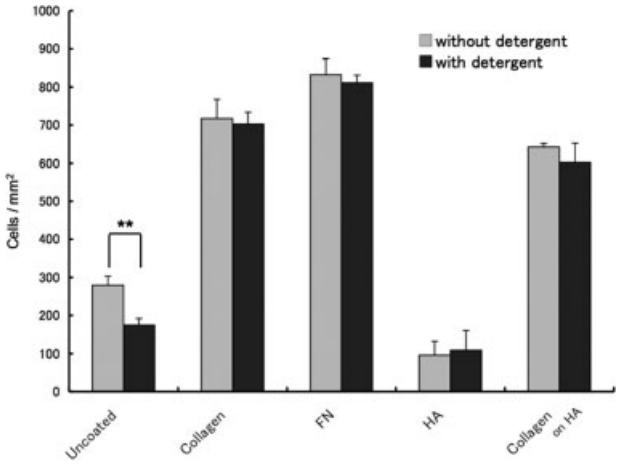

Figure 6. For the fabrication of three-layered parylene-C stencils, a thin film of detergent was deposited between each of the stencil layers. This graph compares cell adhesion on detergent-treated and untreated parylene-C stencils that were subjected to the same sequence of coating with collagen and HA used for dynamic co-culturing. Detergent treatment significantly decreases the number of adhered cells. After coating collagen on top of detergent films, however, cell adhesion is restored to the extent observed on untreated stencils.

red], ALCs [Fig. 7(J–L), orange], NIH-3T3 cells [Fig. 7(P–R), pink], and HL-1 cells [Fig. 7(V–X), blue]. After each co-culture step, the secondary cell type was successfully removed by peeling off the parylene-C membrane. Throughout this process, ES cells remained inside the microwell pattern.

We hypothesized that increased well depths might provide more protection for the ES cells, thereby increasing the number of cells retained after a series of co-cultures. We therefore fabricated parylene-C stencils with 15, 20, and 30 μm depths by changing the heights of the individual parylene-C layers (5–5–5, 5–5–10, and 10–10–10 μm, from top to bottom). We observed that the ES cell patterns in 5–5–5 and 5–5–10 μm stencils degraded to a higher extent than did the patterns in 10–10–10 μm stencils. In the latter, the cell pattern appeared to be robust and stable (Fig. 7). In all stencils, some ES cells were removed from the microwells during the layer peeling steps. In subsequent cell seedings, secondary cell types settled in unoccupied areas inside the microwells, disintegrating the ES cell pattern [Fig. 7(V,W)]. However, 10–10–10 μm stencils exhibited good stability of the ES cell pattern. To quantify the superior performance of 10–10–10 μm stencils, we analyzed the number of retained ES cells per microwell after each step of peeling of the individual parylene-C layers. The number of ES cells retained inside the microwells of 10–10–10 μm stencils was significantly higher ($p < 0.01$) than in the microwells of 5–5–5 μm stencils after each step of peeling (Fig. 8). 5–5–10 μm stencils—stencils in which only the bottom layer was increased in thickness to provide protection to the ES cell pattern—showed improved pattern stability toward the end of the co-culture sequence, leading to a significant increase of retained ES cells during the final co-culture with HL-1 cells (Fig. 8). The 10-μm thick parylene-C layer on the bottom of these stencils retained cells more effectively than the 5-μm thick bottom layer, leading us to conclude that thicker stencils improve the stability of the ES cell pattern. Of the stencil thicknesses used, 10–10–10 μm stencils were found to retain the highest number of cells and thus the most effective design for maintaining a stable cell pattern throughout the dynamic co-culture.

Cellular cross-talk-such as cell–cell interactions and cell communication via soluble factors-has fundamental importance in cell biology. Precise control of the cellular microenvironment and well-defined and flexible co-culture systems are crucial for studying such cross-talk. For this purpose, static patterned co-cultures of two or more cell types have been generated using various approaches.[12,16–22,25] These previous attempts, however, have focused on aspects of primary–secondary cell type interactions and were not amenable to dynamic co-culture. There exist applications for which such a dynamic co-culture would be particularly useful. For example, conditioning ES cells through sequential exposure to various heterotypic cells or secreted factors has been used in stem cell differentiation protocols.[8–10]

In this study, we show that multilayer parylene-C stencils can be used for co-culturing a sequence of at least five different cell types with microscale control over the location of these cells. The described technology is versatile and the temporal aspect of the co-culture can be varied (as can the order of exposure to secondary cell types). Additionally, combined selective patterning of proteins and cells onto individual parylene-C stencils offers the opportunity to match each secondary cell type to an appropriate ECM environment. In the present study, we chose a microwell pattern to localize the ES cells. The pattern and the height of the parylene-C stencil can be varied, perhaps to yield increased interaction with secondary cell types.

CONCLUSIONS

We have developed a microfabricated multilayered parylene-C stencil for engineering the cellular microenvironment. Our stencils can be selectively patterned with different proteins and cells at microscale resolution. We further demonstrate an approach for the generation of dynamic co-cultures of

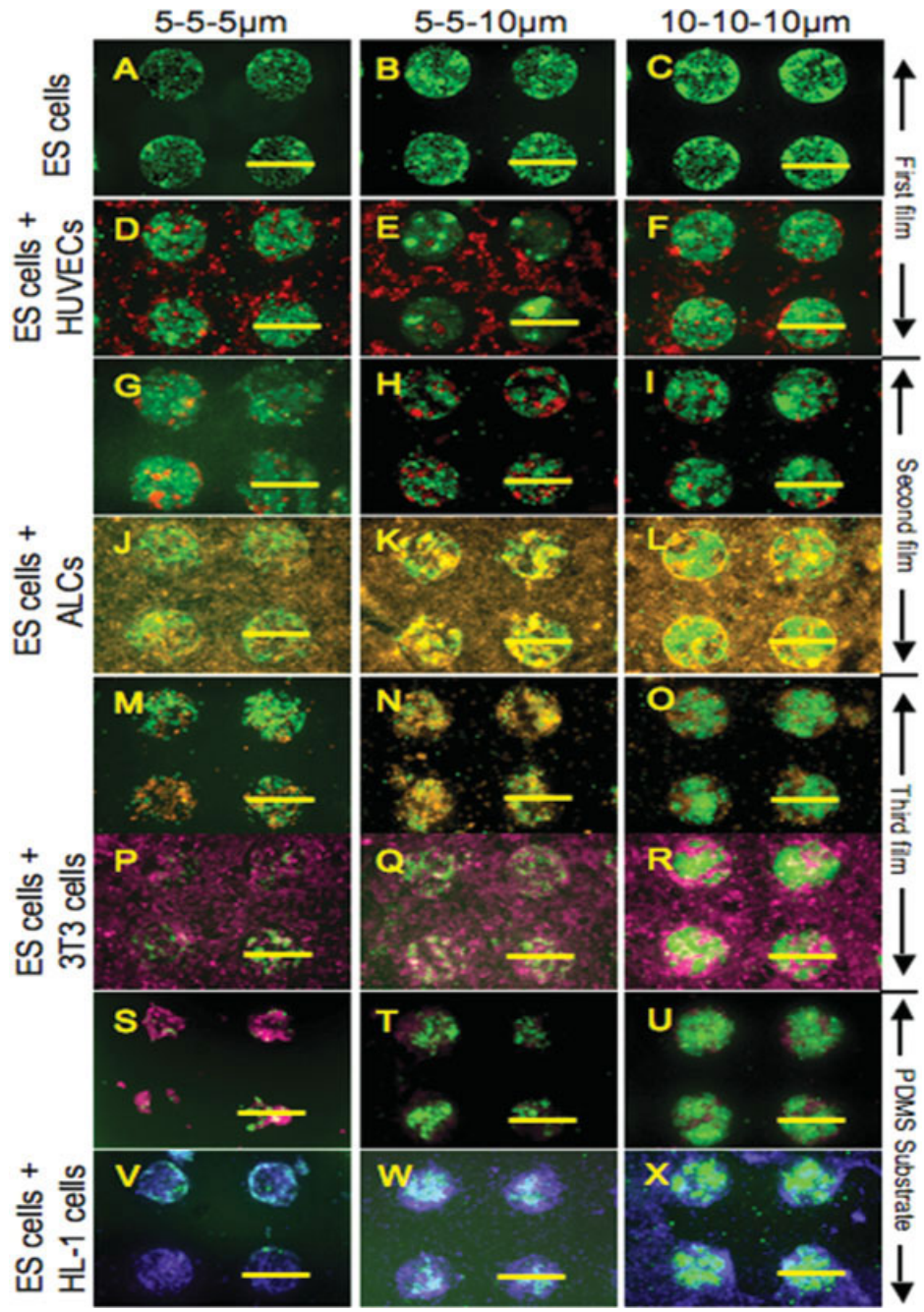

Figure 7. Fluorescent images of the formation of dynamic co-cultures using parylene-C stencils of various thicknesses. HA-coated parylene-C stencils were reversibly sealed on FN-treated PDMS and then seeded with murine embryonic stem (ES) cells (green, A–C). The ES cells were first co-cultured with HUVECs (red, D–F) on top of the first parylene-C layer of the stencil. After peeling away the top layer together with the HUVECs (G–I), ALCs (yellow) were seeded onto the second stencil layer (J–L). After peeling off this second layer, NIH-3T3 cells (pink) were co-cultured (P–R). After removal of the NIH-3T3 cells (S–U), HL-1 cells (purple) were patterned on the exposed PDMS substrate (V–X) for the final co-culture (Scale bars = 200 μm). Rows of images show the co-culture experiment performed with stencils of all three designs, with thicknesses of individual layers (from top to bottom) of 5–5–5, 5–5–10, and 10–10–10 μm. The ES cell pattern inside 10–10–10 μm stencils remained most stable after this sequence of four co-cultures. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

at least five different cell types with spatiotemporal control. The described procedure is flexible and overcomes constraints of existing co-culture systems, which are mostly limited to two different cell types. Furthermore, in our system, selective protein adsorption can be performed in combination with cell patterning to create co-cultures in which each cell type can be matched with an optimal ECM. Our

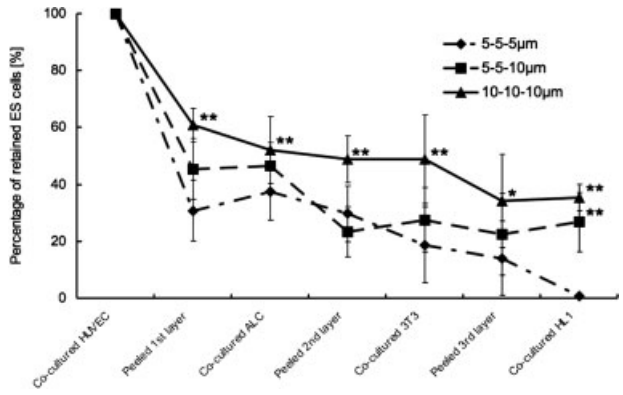

Figure 8. A comparison of the number of retained ES cells in the wells for multilayer stencils with various layer thickness combinations. Retained cell numbers in the parylene-C microwells changed as the stencils with different thickness were utilized (*$p < 0.05$, **$p < 0.01$). There was a statistically significant difference between each of the corresponding points on the 5–5–5 μm and 10–10–10 μm curves. This result indicates that stability of micropatterned cells within parylene-C microwells depends on the stencil layer thickness combination.

technique is simple, versatile, and inexpensive, and it may find potential application in studying stem cell differentiation, developmental processes, wound healing, and pathogenic processes.

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