MULTILAYER PARYLENE-C STENCILS FOR DYNAMICALLY CONTROLLING CELL INTERACTIONS

C.-L. Chen[1], S. Jinno[2,3], H. Möller[2,4], B. Rajalingam[2], S.-H. Chao[1], S. Selvarasah[1], A. Khademhosseini[2,5], and M. R. Dokmeci[1]

1. Electrical and Computer Engineering Department, Northeastern University, Boston, MA, USA.
2. Center for Biomedical Engineering, Department of Medicine, Brigham and Women's Hospital, Harvard Medical School, Boston, MA, USA.
3. Department of Periodontology, Aichi Gakuin University School of Dentistry, Nagoya, Japan.
4. École Supérieure de Biotechnologie de Strasbourg, Strasbourg, France.
5. Harvard-MIT Division of Health Sciences and Technology, Massachusetts Institute of Technology, Cambridge, MA, USA.

ABSTRACT

Most cell processes involve dynamic interactions between the cells and their microenvironment and the ability to control the microenvironment is of crucial importance for studying these processes in-vitro. In this study, we describe a technology for creating multilayer and mechanically robust parylene-C stencils and demonstrate the ability to generate a series of at least 5 temporally controlled cell co-culture micropatterns using embryonic stem cells as the primary cell type. Utilizing layer by layer coatings of collagen and fibronectin, both untreated and detergent treated Parylene surfaces is rendered adhesive for cells. Co-culturing using Parylene stencils may find broad applications in studies investigating cellular interactions and in studies requiring a controlled microenvironment, such as stem cell differentiation.

1. INTRODUCTION

The complex and dynamic interplay of micro-environmental stimuli mediated by the extracellular matrix and cellular cross-talk regulates cell fate decisions, self-renewal, and migration. 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 [1]. 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. Microscale technologies are a potentially powerful method of achieving this complexity as they can used to engineer the cellular microenvironment with high reproducibility and resolution [2].

Patterned co-cultures of two or more cell types have been generated using approaches such as photolithography [3], layer-by-layer deposition of cell-adhesive materials [4], and microfluidics [5]. Despite their usefulness in controlling the cellular interactions these approaches can not be used to control the dynamic cell-cell contact. Recently, Hui and Bhatia have demonstrated a interdigitated comb-like microchip to control the dynamic interactions between hepatocytes and stromal cells [6]. We recently developed a parylene stencil for patterning cells in-vitro [7], yet it could not be applied for the sequential co-culture of more than three cell types. Kuribayashi has developed a parylene-C lift-off process for patterning biomolecules (proteins and lipids) [8], yet the applications to patterning cells were not demonstrated. Although the static co-cultures provide interactions between other cell types, they do not replicate the dynamic aspects of the in vivo environment.

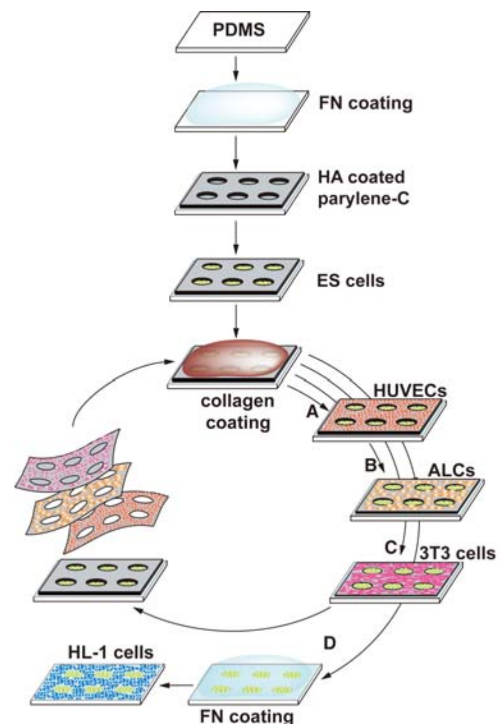

Figure 1: A schematic of the multilayer parylene-C stencil based co-culturing process.

In this study, we develop a multilayer parylene-C stencil technology and demonstrate its potential for patterning extracellular matrix (ECM) proteins and cells. Using the multilayer parylene-C stencil, we describe a novel, rapid, and convenient method for the generation of dynamic co-cultures of 5 different cell types (Fig.1), in which cell-cell, cell-soluble factors and cell-ECM interactions can be controlled in a spatiotemporal manner.

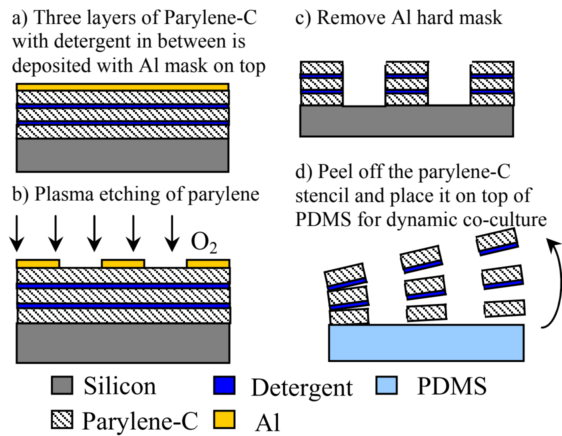

Figure 2: Fabrication process for the multilayer parylene-C stencil.

2. FABRICATION OF MULTILAYER PARYLENE-C STENCILS

A schematic of the fabrication process is illustrated in Fig.2. The process begins by cleaning three-inch silicon wafers in a piranha solution (1:1 H_2SO_4:H_2O_2) for 10 minutes, rinsing and baking them for 10 minutes at 150˚C. The wafers were then coated with hexamethyldisilazane (HMDS) to facilitate removal of the parylene-C stencil stack from the wafer after fabrication. 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). In order to peel the stencil layers individually, an anti-stiction layer (detergent, micro 90) was applied via spin coating prior to the deposition of the subsequent parylene-C layers. Next, a 2000Å thick aluminum layer was deposited (Fig.2a) and patterned to serve as a hard mask. Microwells for patterning cell clusters were created on the parylene stencil utilizing a low temperature (5°C) dry etch process (Fig.2b) in an Inductively Coupled Plasma (ICP) reactor (Plasmatherm 790) [9]. After etching, the Aluminum hard mask was removed in PAN Al etchant at 50°C for 2 min (Fig.2c).

We fabricated multilayer stencils with layers that have 3 different thicknesses (5-5-5, 10-10-10, and 5-5-10 µm). SEM micrographs of the multilayer stencils are shown in Fig.3. In our previous study, we have found that 200 µm diameter microwells were suitable for culturing various types of cells, and hence microwells with this diameter were chosen for the current study [7] (Fig.3A). Cross sectional view shows that the fabricated stencils are comprised of three individual layers that can be independently separated from each other (Fig.3B). Utilizing a low temperature plasma etch process enabled us to achieve vertical side walls which was crucial for realizing the desired dimensions since the stencils were relatively thick (up to 30 µm).

Different anti-stiction materials have been tried between the individual stencil layers which included parafilm®, polydimethylsiloxane (PDMS), and detergent.

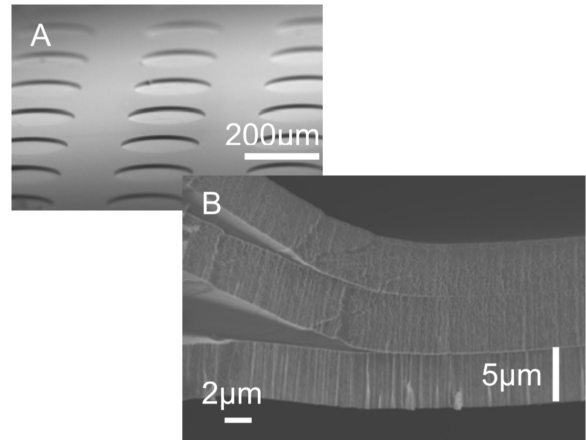

Figure 3: SEM micrographs of multilayer parylene-C stencils A) top view, and B) cross-sectional view.

An ideal anti-stiction layer should be easy to etch, biocompatible, and transparent since most cell experiments utilize inverted microscopes. Among these materials, parafilm® was too thick (several hundred µm) to etch. PDMS was biocompatible and its thickness can be reduced down to 20 µm, yet ICP dry etching of PDMS was fairly slow. Kuribayashi had tried Aluminum as the anti-stiction layer [8], which worked well except it was not transparent. Detergent was also tried in their study, yet since detergent was not biocompatible, cell patterning was not attempted. Furthermore, utilizing a layer by layer coating process [4], one can potentially convert a detergent coated Parylene surface into a cell adhesive surface. To explore this further, we compared the adhesion of NIH-3T3 (fibroblasts) cells on uncoated and detergent-coated parylene-C stencils. Stencils were also treated with other ECM components used for the generation of cell cultures (collagen, fibronectin, HA, and layer-by-layer deposition of collagen on HA) with the results summarized in Fig.4.

Cell adhesion to untreated Parylene surfaces are at a moderate level, yet with the application of collagen and fibronectin (FN) it can be significantly enhanced (Fig.4). Application of Hyaluronic Acid (HA) reduces non-specific cell adhesion to surfaces and was also demonstrated on the Parylene surface. Furthermore, the layer by layer deposition

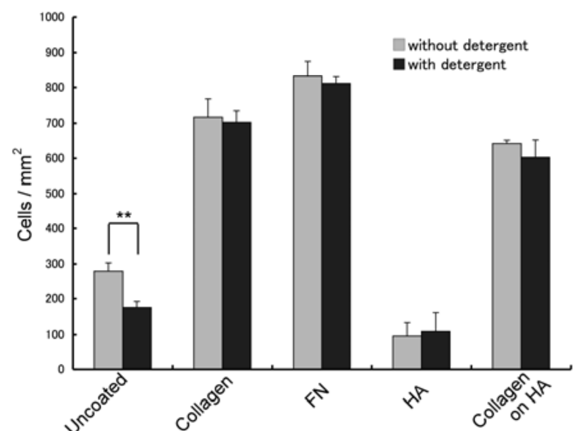

Figure 4: Cell adhesion on untreated and detergent-treated parylene-C stencils coated with ECM factors.

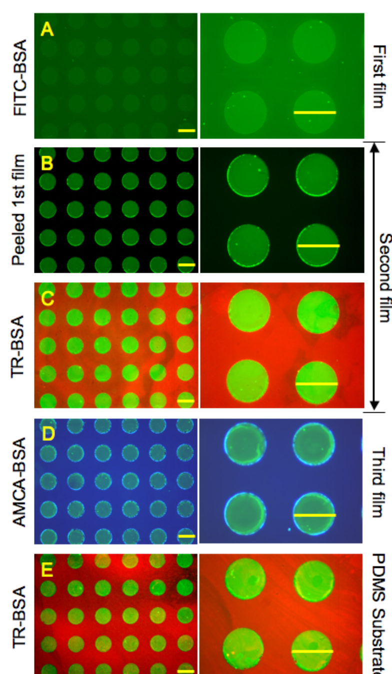

Figure 5: Patterning of fluorescently labeled proteins using a multilayer stencil, scale bar is 200 µm.

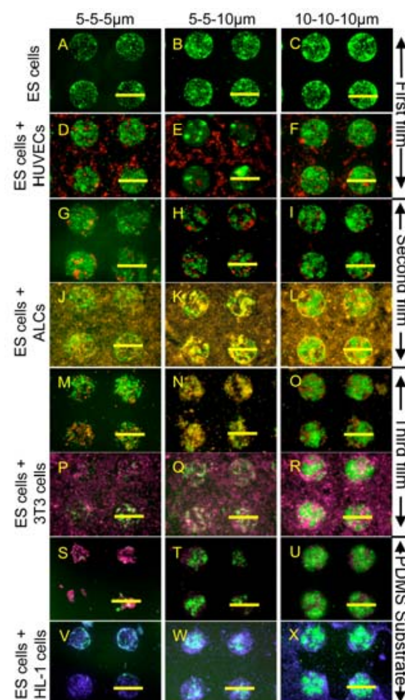

Figure 6: Fluorescent images of the cells during formation of dynamic co-cultures, scale bar is 200 µm.

of collagen on to the HA coated parylene surface rendered a surface which was cell adhesive. In almost all cases, the detergent treatment of a surface reduced the amount of cell adhesion on to that surface, furthermore the application of collagen and FN switched the surface to a cell adhesive one. We believe that the surface treatment with collagen and FN improves cell adhesion by forming a layer above the detergent film, and the presence of the biocompatible ECM molecules hence attenuates the observed effect of detergent treatment on cell adhesion.

3. RESULTS AND DISCUSSION

We have designed and fabricated multilayer parylene-C stencils which consisted of three individually peelable layers of parylene-C (the thicknesses ranged from 5 to 10 µm). After fabrication, the stencils were removed from the substrates and were utilized in patterning proteins and cells and these results will be discussed next.

A) Patterning of multiple proteins

Protein patterning is utilized to generate substrates that are favorable for cell adhesion. Hence, we initially seal the multilayer parylene-C stencils on a PDMS surface and demonstrate successful patterning of fluorescently labeled BSA. First, both the stencil stack and the PDMS substrate exposed through the microwells were coated with 100 µg/ml fluorescein isothiocyanate (FITC) coupled to BSA (Fig.5A). The top stencil layer was peeled off to yield a FITC-BSA protein pattern inside the microwells (Fig.5B). Next, the surface was patterned with 50 µg/ml Texas Red-BSA (TR-BSA) to yield a co-pattern with the FITC-BSA (Fig.5C). The middle stencil layer was then peeled off, and the bottom stencil layer was coated with 50 µg/ml 6-((7-Amino-4-methylcoumarin-3-acetyl)amino)hexanoic acid coupled to BSA, yielding another protein co-pattern (Fig.5D). Finally, after removal of the bottom stencil, the PDMS surface was patterned with 50 µg/ml TR-BSA (Fig.5E). As seen in these figures, the proteins can be selectively patterned with microscale resolution using parylene-C stencils. Selective patterning of proteins such as antibodies and enzymes has recently attracted much interest for the study of specific protein-protein interactions and the development of diagnostic kits and protein sensors, hence the Parylene-C stencil technology might find applications in the rapidly developing field of selective protein patterning.

B) Dynamic co-culturing on parylene-C stencils and the stability of cell patterns

To study patterning and dynamic co-culturing of cells, we next performed cell seeding experiments utilizing the multilayer parylene-C stencil. Dynamic co-cultures of 5 different cell types (Pluripotent murine embryonic stem (ES), Human Umbilical Vein Endothelial Cells (HUVEC), Murine epithelial Ameloblast-Lineage Cells (ALC), NIH-3T3s, and HL-1 cells) were generated (Fig.1). First, an HA coated multilayer parylene-C stencil stack was reversibly sealed on a FN treated PDMS substrate and ES cells were cultured inside microwells (Fig.6A). Next, a layer by layer deposition approach using collagen was applied to restore the cell adhesive properties to the top stencil surface. HUVEC cells were then brought in and co-cultured with ES cells (Fig.6D) and after this culturing step, the top stencil layer was gently peeled off (Fig.6G). A layer of collagen layer was subsequently deposited on the stencil. The third cell type (ameloblasts) was next seeded on the stencil (Fig.6J) and after 4 hours of culturing, the middle stencil layer was removed

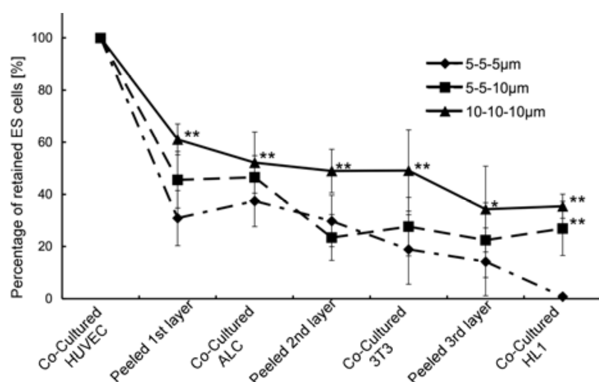

Figure 7: The number of remaining ES cells inside the microwells for different stencil thicknesses.

(Fig.6M). To enhance cell adhesiveness, another layer of collagen was applied on the stencil. The fourth cell type (NIH-3T3's) was next seeded on the stencil (Fig.6P) and after co-culturing 3T3's with ES cells (Fig.6S), the bottom stencil was removed. FN was then applied on to the PDMS substrate housing the ES cells and subsequently HL-1 cells were seeded (Fig.6V). The fluorescent images (shown in Fig.6) indicate that utilizing the multi layer stencil technology allows co-culturing of 5 different cell types and the cells survive the stencil removal and handling processes.

We hypothesized that increasing the thickness of the stencils would enhance the stability of the microwells thereby increasing the number of ES cells retained (inside the microwells) during stencil removal and handling processes. We then fabricated parylene-C stencils with different individual layer thicknesses (5-5-5, 5-5-10, and 10-10-10 µm, from top to bottom). We observed that the ES cell micropatterns inside 5-5-5 and 5-5-10 µm stencils degraded to a higher extent than did the patterns in 10-10-10 µm thick stencils. In the latter, the cell patterns appeared to be robust and stable (Fig.6). In all cases, some ES (primary) cells were removed from the microwells during the stencil peeling steps. In subsequent cell seedings, secondary cell types settled in unoccupied areas inside the microwells, disintegrating the ES cell pattern (Fig.6V, W). However, 10-10-10 µm stencils exhibited good stability for the ES cell patterns. To quantify the performance of 10-10-10 µm stencils, we counted the number of retained ES cells per microwell after peeling off each stencil layer. The number of ES cells retained inside the microwells for the 10-10-10 µm thick stencils was significantly higher ($p<0.01$) than in the microwells for the 5-5-5 µm thick stencils as shown in Fig.7. 5-5-10 µm stencils--stencils in which only the bottom layer was increased in thickness to provide protection for the ES cell patterns--showed improved pattern stability towards the end of the co-culture sequence, leading to a significant increase in retained ES cells during the final co-culture with HL-1 cells (Fig.7). The bottom stencils that were 10 µm in thickness were more effective in retaining the ES cells compared to the 5 µm thick ones, leading us to conclude that thicker stencils improve the stability of the ES cell patterns. In summary, 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 preserving a stable cell pattern throughout the dynamic co-culture experiments.

4. CONCLUSIONS

We have developed a multilayer parylene-C stencil technology for creating microscale patterns of proteins and cells. We demonstrate that the multilayer Parylene stencils can be used to generate dynamic co-cultures of at least 5 different cell types with a spatio-temporal control. The procedure is flexible and overcomes constraints of existing co-culture systems, which are mostly limited to two different cell types. In this study, a circular microwell pattern with a diameter of 200 µm was utilized for housing the primary (ES) cells. The geometry and the thickness of the parylene-C stencils can be engineered to enhance/reduce the interactions with the secondary 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 technique is simple, versatile, and inexpensive, and it may find potential application in studying stem cell differentiation, developmental biology, and regenerative medicine.

ACKNOWLEDGMENTS

This paper was supported by the Coulter Foundation, National Institutes of Health (NIH), the Center for Integration of Medicine and Innovative Technology (CIMIT), the Institute for Soldier Nanotechnology (ISN), and the Charles Stark Draper Laboratory.

REFERENCES

[1] P. W. Zandstra and A. Nagy, "Stem cell bioengineering," *Annu Rev Biomed Eng,* vol. 3, pp. 275-305, 2001.

[2] A. Khademhosseini, R. Langer, J. Borenstein, and J. P. Vacanti, "Microscale technologies for tissue engineering and biology," *PNAS,* vol. 103, pp. 2480-7, 2006.

[3] S. N. Bhatia, M. L. Yarmush, and M. Toner, "Controlling cell interactions by micropatterning in co-cultures: hepatocytes and 3T3 fibroblasts," *J. Biomed. Mater. Res.,* vol. 34, pp. 189-99, 1997.

[4] A. Khademhosseini, K. Y. Suh, J. M. Yang, G. Eng, J. Yeh, S. Levenberg, and R. Langer, "Layer-by-layer deposition of hyaluronic acid and poly-L-lysine for patterned cell co-cultures," *Biomaterials,* vol. 25, pp. 3583-92, 2004.

[5] S. Takayama, J. C. McDonald, E. Ostuni, M. N. Liang, P. J. Kenis, R. F. Ismagilov, and G. M. Whitesides, "Patterning cells and their environments using multiple laminar fluid flows in capillary networks," *PNAS,* vol. 96, pp. 5545-8, 1999.

[6] E. E. Hui and S. N. Bhatia, "Micromechanical control of cell-cell interactions," *PNAS,* vol. 104, pp. 5722-6, 2007.

[7] D. Wright, B. Rajalingam, S. Selvarasah, M. R. Dokmeci, and A. Khademhosseini, "Generation of static and dynamic patterned co-cultures using microfabricated parylene-C stencils," *Lab Chip,* vol. 7, pp. 1272-9, 2007.

[8] K. Kuribayashi, Y. Hiratsuka, T. Yamanura, and S. Takeuchi, "Sequential parylene lift-off process for selective patterning of biological materials," in *Proc., 20th Int. MEMS Conf.,*, Kobe, Japan, 2007, pp. 501-4.

[9] S. Selvarasah, S.-H. Chao, C.-L. Chen, D. Mao, J. Hopwood, S. Ryley, S. Sridhar, A. Khademhosseini, A. Busnaina, and M. R. Dokmeci, "A high aspect ratio, flexible, transparent and low-cost parylene-C shadow mask technology for micro patterning applications," in *Proc., 14th Int. Conf. on Solid-State Sensors, Actuators and Microsystems (Transducers '07)* vol. 1 Lyon, France, 2007, pp. 1019-1022.