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Molecular Etching – A Novel Methodology for the Generation of Complex Micropatterned Growth Surfaces for Human Cellular Assays

Ayhan Atmanli,

Cardiovascular Research Center Massachusetts General Hospital 185 Cambridge Street, Boston, MA 02114, USA

Harvard Medical School 250 Longwood Avenue, Boston, MA 02115, USA Dr. Ibrahim J. Domian

Dongjian Hu, and

Cardiovascular Research Center Massachusetts General Hospital 185 Cambridge Street, Boston, MA 02114, USA

Harvard Medical School 250 Longwood Avenue, Boston, MA 02115, USA Dr. Ibrahim J. Domian

Ibrahim J. Domian*

Cardiovascular Research Center Massachusetts General Hospital 185 Cambridge Street, Boston, MA 02114, USA

Harvard Medical School 250 Longwood Avenue, Boston, MA 02115, USA Dr. Ibrahim J. Domian

Harvard Stem Cell Institute 1350 Massachusetts Avenue Cambridge, MA 02138, USA

Abstract

The extracellular matrix (ECM) is the non-cellular component of all tissues consisting of many different bioactive macromolecules including proteins, proteoglycans, glycoproteins and gradients of growth factors. It is a highly complex and dynamic structure that is subject to constant remodeling *in vivo*. The ECM not only provides essential structural support for tissues and cell layers but also modulates molecular and biomechanical signaling cues.^[1-4] ECM composition is tightly regulated during normal development and hemostasis and varies with tissue type as well as developmental stage. Hearts of different developmental stages have significant differences in ECM composition and elasticity.^[5, 6] Dysregulation of the ECM has also been shown to result in human aortic and connective tissue diseases.^[7] In addition, ECM has been shown to control cell behavior and function through its elasticity, topography, and dimensionality. *In vitro*, culture surface stiffness has been shown to directly control the lineage commitment of mesenchymal stem cells.^[8, 9]

*domian@mgh.harvard.edu.

Supporting Information

Supporting Information is available online from the Wiley Online Library or from the author.

Keywords

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Accordingly, the *in vitro* recapitulation of *in vivo* physiology and pathophysiology requires culturing cells of interest on the biologically relevant ECM on surfaces of physiologic stiffness. Recent work from a number of laboratories has highlighted the importance of microenvironmental cues in controlling cell morphology. Conventional *in vitro* cell culturing techniques lack the geometric cues normally present *in vivo*. As a result of this consideration, a number of methods to control cell morphology *in vitro* have been developed. These include biological and synthetic scaffolds^[10-12] as well as micropatterned two-dimensional tissues^[13-15] that can be layered into three-dimensional tissues.^[16] Microcontact Printing (μ CP) is the mainstay technique used to control cell morphology and engineer micropatterned two-dimensional growth surfaces *in vitro*. The technique has been widely used to micropattern cardiac myocytes into anisotropic two-dimensional tissue, for example.^[13, 14, 17, 18] In addition, μ CP has been used to study the behavior of neuronal cells,^[19] skeletal muscle cells,^[19] vascular smooth muscle cells,^[20] endothelial cells^[21] and hepatocytes^[22]. Alternative micropatterning techniques include microfluidics,^[23] photomask-assisted patterning,^[24] photo-,^[25] laser-^[26] and electron beam-lithography.^[27] These micropatterning techniques have mainly been limited to single ECM proteins that are not specific to the native tissue of the cell type to be seeded. Given the complexity of the ECM *in vivo*, it is desirable to provide cells *in vitro* complex rather than single extracellular cues in order to mimic the *in vivo* ECM microenvironment.

Thus, despite the widespread use of the μ CP technology in generating micropatterned cellular growth surfaces, several obstacles limit its generalizability to different culture surfaces and different ECM proteins. The micropatterning of complex biologically active ECM mixtures through μ CP, for example, depends on the capacity of these, proteins to adsorb on the PDMS stamp and to then transfer to the culture surface of choice. Since different proteins have different chemical properties, the efficiency with which they bind to the PDMS stamp and transfer to the culture surface will necessarily vary. As a result, μ CP cannot be used to micropattern complex ECM protein mixtures in a stoichiometrically faithful manner. Furthermore, since micropatterned proteins must retain their biological activity to be biologically useful, a previous report showing that μ CP can result in diminished activity of transferred proteins further limits the utility of this approach.^[28] As a result, examining the printed biologically relevant ECM proteins necessitates the development of novel approaches that allow micropatterning of complex protein mixtures with full biological activity. In order to address this need, we have developed a novel methodology, termed Molecular Etching (ME), to directly micropattern surface-bound complex ECM.

To rigorously define the efficiency of transfer of active ECM proteins using direct binding and μ CP approaches, we performed direct comparative analyses of these two ECM deposition techniques. We first directly bound Fibronectin (FN), Collagen Type I (COL I),

Laminin (LAM) and Matrigel (MG) onto PDMS surfaces. We controlled for the μ CP process by establishing conformal contact with an uncoated PDMS stamp. In parallel, we also microcontact printed ECM proteins onto PDMS surfaces using a flat stamp. We plated human Embryonic Stem Cell-derived Cardiac Myocytes (hESC-CMs) on the culture surfaces and observed robust attachment of hESC-CMs plated on FN independent of whether FN had been directly bound or microcontact printed onto the culture surface. In contrast, we observed robust cellular attachment on directly bound COL I, LAM and MG surfaces but reduced or minimal cell attachment on the microcontact printed surfaces (**Figure 1A**). We then further validated these results by plating hESC-CMs on surfaces micropatterned with these four ECM proteins by ME and μ CP (**Figure 1B**) and showed improved patterning and attachment of cells plated on ME versus μ CP surfaces.

These results suggest that, when compared to direct binding of ECM proteins, μ CP can result in either reduced amount or reduced activity of transferred ECM proteins. Although previous work has allowed for the successful μ CP of FITC-labeled COL I,^[29] it is possible that the transfer efficiency of fluorescently-labeled COL I by μ CP is below the threshold cells need to attach *in vitro* but above the fluorescence detection limit. To determine if ECM printing leads to lower surface concentration of proteins compared to direct binding, we performed a stoichiometric analysis of proteins deposited onto PDMS surfaces with both techniques. A protein mixture consisting of FN labeled with Alexa Fluor 488 (FN-488) and Laminin (LAM) labeled with Alexa Fluor 647 (LAM-647) and unlabeled COL I was deposited onto PDMS surfaces through direct binding. We controlled for μ CP by establishing conformal contact with an uncoated PDMS stamp. Concurrently, we also microcontact printed the same protein mixture onto PDMS surfaces using a flat stamp. We then immunofluorescently stained COL I with Alexa Fluor 546 secondary antibody and quantified the fluorescence intensity of each protein on both surfaces (**Figure 1C**). We found that FN is deposited onto PDMS surfaces with the same efficiency by μ CP as by direct binding. LAM and COL I, however, showed a diminished printing efficiency ($55.1 \pm 9.3\%$ and $25.0 \pm 1.3\%$, respectively) resulting in altered protein concentration and, critically, an altered stoichiometry of the deposited proteins. This limitation seriously undermines the use of μ CP for the deposition of micropatterned complex biologically relevant ECM protein mixtures since differences in printing efficiency result in different stoichiometry of the deposited proteins when compared to the starting material.

We next investigated whether μ CP may alter protein activity. We performed an enzyme kinetics assay comparing directly bound to microcontact printed Proteinase K on PDMS surfaces. It has been reported that Proteinase K follows Michaelis-Menten kinetics.^[30] We obtained the Michaelis constant K_m for Proteinase K deposited through both investigated methods. K_m is independent from the enzyme concentration on the surface and is so a reliable parameter to compare the enzyme function after binding and printing, respectively. **Figure 1D** displays the results with the double-reciprocal Lineweaver-Burk plot. We found that the K_m value for microcontact printed Proteinase K is 4-fold higher compared to directly bound Proteinase K indicating that the protein affinity towards its substrate has been significantly diminished after printing.

To determine if there are any differences in the deposition of proteins, we then used Atomic Force Microscopy (AFM) to determine the surface topography of directly bound versus microcontact printed ECM. Matrigel (MG) is a complex ECM mixture isolated from the Engelbreth-Holm-Swarm tumor and is widely used in stem cell maintenance culture and stem cell directed differentiation protocols. MG was either directly bound or microcontact printed on PDMS surfaces. AFM analysis revealed that MG directly bound to the culture surface adopted a relative flat and homogeneously distributed protein layer. In contrast, microcontact printed MG showed a rough, uneven surface topography with areas of both abundant and sparse protein distribution, resulting in at least 5nm height difference of attached proteins (**Figure 1E**). The non-uniform appearance of proteins on microcontact printed surfaces may be attributed to the formation of supramolecular protein structures that may result from protein aggregation. In turn, protein aggregates may have diminished or altered activity.^[31]

Collectively, these results strongly argue that hESC-CMs have a reduced attachment to microcontact printed surfaces due to a significant reduction in the deposition of biologically active ECM proteins when compared to direct binding. As a result, direct binding of ECM proteins provides more homogeneous growth surfaces for Tissue Engineering purposes compared to printed ECM. We therefore sought to develop a novel methodology that would allow us to capitalize on the superior surface chemistry characteristics of direct protein binding and at the same time to micropattern the bound ECM proteins. This methodology, termed Molecular Etching (ME), is summarized in **Scheme 1**. μ CP involves a two-step process where the protein being patterned is first adsorbed on a micropatterned PDMS surface and then transferred onto a growth surface by conformal contact between the stamp and the growth surface. In contrast, ME eliminates the need for solid-phase transfer of proteins from the PDMS stamp to the culture surface. Instead, the protein is directly bound to the culture surface by either adsorption or covalent binding. This allows for the homogenous distribution of active protein on the culture surface. Conformal contact with a protease-coated micropatterned PDMS stamp is then used to degrade the directly bound protein in the areas of contact. This leaves a micropatterned growth surface and at the same time eliminates the dependency of the ECM/PDMS surface chemistry for protein transfer. ECM proteins can thereby be micropatterned in their native conformation without the need for chemical modifications such as those previously used to micropattern Collagen Type I, for example.^[32] In principle, using ME technique, any ECM can be micropatterned onto any surface provided that the ECM has been successfully deposited onto the growth surface and an appropriate protease has been identified. Notably, proteins covalently bound onto the growth surface can be micropatterned with ME providing a reliable micropatterning technique and at the same time ensuring the presence of all proteins deposited onto the surface.

As shown in Figure 2, we then demonstrated the generalizability of ME by micropatterning a variety of growth surfaces commonly used for cell culture purposes (including Tissue Culture Polystyrene (TCPS), Polydimethylsiloxane (PDMS), Glass and Polyacrylamide) with biologically active purified ECM proteins (including FN, COL I and LAM) or complex

ECM protein mixture (MG). **Table 1** summarizes the optimum conditions for each protein and protease used in this study.

Recent evidence from a number of laboratories demonstrated that ECM proteins could have profound physiological effects on cells *in vitro*.^[33-35] In order to test the significance of varying ECM proteins on the behavior of cardiac myocytes, we examined the effects of the abovementioned four ECM proteins on hESC-CMs. To replicate physiologic conditions, we plated hESC-CMs on PDMS surfaces (PDMS₅₂₇) that were soft enough to allow the cells to shorten (~10-20kPa).^[19] After initial attachment, cells were fixed and stained with sarcomeric α -actinin antibody. This allowed the fraction of cardiac myocytes to be quantified (**Figure 3A and B**). Surprisingly, we found that cells cultured on LAM- and MG-coated surfaces had the highest proportion of cardiac myocytes ($67\pm15\%$ and $48\pm21\%$, respectively) whereas cells cultured on COL I had the lowest proportion of attached cardiac myocytes ($14\pm4\%$). We found that $34\pm8\%$ of attached cells on FN-coated surfaces were cardiac myocytes. We then generated micropatterned surfaces with these ECM proteins on PDMS₅₂₇ surfaces using our ME approach. hESC-CMs were plated on 30 μ m lines and stained with sarcomeric α -actinin and Connexin 43 antibodies (**Figure 3C**). hESC-CMs cultured on all four micropatterned ECM proteins were able to adopt a rod-like shape with aligned sarcomeres on molecular etched surfaces. In addition, gap junctions localized at intercellular junctions, suggesting electrical coupling between cardiac myocytes.

The defining characteristic of cardiac myocytes is their capacity to contract. As a result, fractional shortening has been used as a key parameter of cardiac myocyte function and contractility.^[36-38] In order to determine the effect of different ECM proteins on the fractional shortening of cardiac myocytes, we generated PDMS₅₂₇ surfaces with micropatterned ECM proteins. This allowed us to directly compare the fractional shortening of rod-like cardiac myocytes cultured on micropatterned FN, COL I, LAM and MG single cell islands with 100 $\times$ 30 μ m dimensions. As shown in **Figure 3D**, hESC-CMs cultured under these conditions adopted an aligned rod-like shape. We then quantified the fractional shortening 4 and 7 days after seeding on molecular etched surfaces (**Figure 3E**). Of great interest, we found that cells cultured on different ECM proteins had different degrees of fractional shortening. The largest fractional shortening points on MG- and COL I-coated surfaces. Interestingly, COL I resulted in the least favorable surface for cardiac myocyte attachment but a very high degree of fractional shortening. In contrast, LAM provided a high degree of cellular attachment but poor fractional shortening. MG, a complex ECM protein mixture that predominantly consists of LAM and Collagen Type IV, resulted in both high efficiency of cellular attachment as well as a high degree of fractional shortening. These results collectively point to the necessity of recapitulating natural ECM composition and activity to allow for optimum assays of cellular function. Future studies can now be geared at evaluating the role of cardiac developmental stage-specific ECM (including ECM from adult hearts^[39]) in coordinating the development and cellular function of aligned cardiac myocytes on surfaces with physiological stiffnesses. Furthermore, single cell assays could be developed using ME to study the effect of physiologic as well as pathologic ECM on cardiac myocytes as well as other cell types.

In conclusion, we have developed a novel methodology to reliably micropattern complex, biologically active ECM protein mixtures on a variety of cell growth surfaces. Our methodology allows for surface-bound proteins to be directly micropatterned without altering their physical and chemical properties. We also provided data suggesting that complex ECM provide more physiologic cues than single purified proteins. These observations may have further implications with respect to the design of cellular assays that recapitulate cell physiology and pathophysiology for drug discovery and development. This tool could prove powerful in cardiovascular drug discovery since medical therapy remains the mainstay therapy for patients with heart disease. In addition, our approach could prove powerful not only in cardiovascular research but also in other fields where cell shape and cellular interaction with ECM proteins are important. In tumor biology, for example, the role of tumor-specific ECM on tumor cell biology could be examined in a highly controlled manner where both cell shape and ECM composition can be controlled with a high degree of precision. Similarly, geometrically confined neuronal networks for biomedical approaches and neuronal cell biology studies could now be grown on specific complex ECM. Of great importance, these approaches are readily scalable for use in automated high-throughput applications.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Comparison of directly bound to microcontact printed ECM

A hESC-derived CMs were seeded on PDMS surfaces directly bound or control microcontact printed (μCP) with Fibronectin (FN), Collagen Type I (COL I), Laminin (LAM) and Matrigel (MG). **B** hESC-derived CMs were seeded on PDMS surfaces micropatterned with FN, COL I, LAM and MG by Molecular Etching (ME) or μCP . **C** A mixture of FN-488, LAM-647 and COL I (subsequently stained with Alexa Fluor 546) was directly bound or microcontact printed on PDMS surfaces. The fluorescence intensity of each protein was then quantified (mean average $\pm$ s.d., $n = 3$; direct binding versus μCP , FN $p = 0.13$, LAM $p = 0.03$, COL I $p = 0.003$). **D** Lineweaver-Burk plots of directly bound and microcontact printed Proteinase K (mean average $\pm$ s.d., $n = 2$; direct binding versus μCP , $p = 0.01$). **E** AFM images of PDMS surfaces directly bound or microcontact printed with MG. Arrows indicate areas with abundant (open arrows) and sparse (closed arrow) amounts of ECM. Scale bar $50\mu\text{m}$. * $p < 0.05$ and ** $p < 0.01$.

Scheme 1. Schematic overview of Microcontact Printing and Molecular Etching

With Microcontact Printing, the protein being patterned is first adsorbed on a micropatterned PDMS surface and then transferred onto a growth surface by establishing conformal contact between the stamp and the growth surface. With Molecular Etching, the ECM is first adsorbed or conjugated onto the culture surface. A micropatterned PDMS stamp coated with an ECM-specific protease is used to establish conformal contact between the stamp and the growth surface. The ECM is specifically degraded at the contact areas.

Figure 2. Generalizability of Molecular Etching

Tissue Culture Polystyrene (TCPS), Glass, Polydimethylsiloxane (PDMS) and Polyacrylamide surfaces were micropatterned with Fibronectin (FN), Collagen Type I (COL I), Laminin (LAM) and Matrigel (MG) proteins using Molecular Etching technique resulting in protein lanes of 30 μ m width. Scale bar 100 μ m.

Figure 3. Effect of selected ECM on hESC-CMs

A hESC-CMs were plated on PDMS₅₂₇ surfaces coated with selected ECM and the fraction of cardiac myocytes was quantified (mean average $\pm$ s.d., $n = 3$; FN versus COL I $p = 2 \times 10^{-6}$, FN versus LAM $p = 2 \times 10^{-5}$, FN versus MG $p = 0.1$, COL I versus LAM $p = 1 \times 10^{-8}$, COL I versus MG $p = 2 \times 10^{-4}$, LAM versus MG $p = 0.04$). **B** Representative images of (A) with immunostaining against sarcomeric α -actinin (green) and DNA (blue). Scale bar 100 μ m. **C** hESC-CMs were plated on PDMS₅₂₇ surfaces molecular etched with selected ECM consisting of 30 μ m wide lines and stained against sarcomeric α -actinin (green), Connexin 43 (red) and DNA (blue). Scale bar 50 μ m. **D** hESC-CMs were plated on PDMS₅₂₇ surfaces molecular etched with selected ECM consisting of single cell islands with 100 $\times$ 30 μ m dimensions. Scale bars 50 μ m. **E** Quantification of fractional shortening at different time points after seeding hESC-CMs on the different ECM-coated PDMS surfaces (mean average $\pm$ s.d., $n = 7-9$ cells; day 4: FN versus COL I $p = 0.02$, FN versus LAM $p = 0.73$, FN versus MG $p = 0.003$, COL I versus LAM $p = 0.01$, COL I versus MG $p = 0.53$, LAM versus MG $p = 0.001$; day 7: FN versus COL I $p = 0.04$, FN versus LAM $p = 0.20$, FN versus MG $p = 0.002$, COL I versus LAM $p = 0.17$, COL I versus MG $p = 0.35$, LAM versus MG $p = 0.01$). * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

Table 1

Surface and Stamp Binding Conditions for Molecular Etching

ECM	Etched Surface	ECM Binding to Surface	Etching Protease	Protease Binding to Stamp
FN	TCPS			
	Glass	Passive adsorption	Proteinase K	Passive adsorption
	PDMS Polyacrylamide	Covalent binding		
COLI	TCPS			
	Glass	Passive adsorption	Collagenase IV	Covalent binding
	PDMS Polyacrylamide	Covalent binding		
LAM	TCPS			
	Glass	Passive adsorption	Proteinase K	Passive adsorption
	PDMS Polyacrylamide	Covalent binding		
MG	TCPS			
	Glass		Proteinase K + Collagenase	
	PDMS Polyacrylamide	Covalent binding		Covalent binding