Patterned Co-Culture of Primary Hepatocytes and Fibroblasts Using Polyelectrolyte Multilayer Templates

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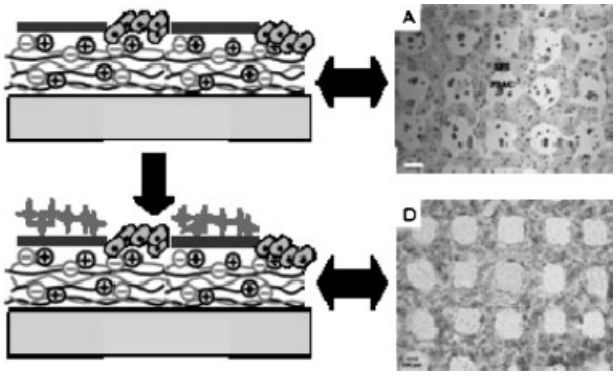
This paper describes the formation of patterned cell co-cultures using the layer-by-layer deposition of synthetic ionic polymers and without the aid of adhesive proteins/ligands such as collagen or fibronectin. In this study, we used synthetic polymers, namely poly(diallyldimethylammonium chloride) (PDAC) and sulfonated polystyrene (SPS) as the polycation and polyanion, respectively, to build the multilayer films. We formed SPS patterns on polyelectrolyte multilayer (PEM) surfaces either by microcontact printing PDAC onto SPS surfaces or vice-versa. To create patterned co-cultures on PEMs, we capitalize on the preferential attachment and spreading of primary hepatocytes on SPS as opposed to PDAC surfaces. In contrast, fibroblasts readily attached to both PDAC and SPS surfaces, and as a result, we were able to obtain patterned co-cultures of fibroblast and primary hepatocytes on synthetic PEM surfaces. We characterized the morphology and hepatic-specific functions of the patterned cell co-cultures with microscopy and biochemical assays. Our results suggest an alternative approach to fabricating controlled co-cultures with specified cell–cell and cell–surface interactions; this approach provides flexibility in designing cell-specific surfaces for tissue engineering applications.



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Introduction

Recent developments in the field of tissue engineering and biomaterials have brought notable advances in culturing cells on bioactive surfaces to guide and control their assembly into functional tissues. Tissue formation and function *in vivo* are influenced by many factors, including cytokines, cell–matrix interactions, topology, mechanical forces, and cell–cell interactions. An important aspect in tissue formation and function is the interaction between the multiple types of cells within the tissue.[1] Mimicking, *in vitro*, this complexity and function is difficult using

Macromol. Biosci. **2007**, *7*, 344–353

DOI: 10.1002/mabi.200600205

traditional co-culture techniques, wherein multiple cell types are seeded randomly. Therefore, regenerating or replacing damaged tissue using *in vitro* strategies has primarily focused on manipulating the cellular environment by modulating the cell-extracellular matrix (ECM) and cell–cell interactions.[2] A challenge in engineering *in vitro* liver tissue is identifying a set of minimal environmental signals required to maintain function for extended periods.[3]

Primary hepatocytes are anchorage-dependent liver cells and, therefore, require a substratum to survive and function. Primary hepatocytes, unlike many other cell types, exhibit more selective behavior *in vitro*, preferentially attaching and spreading on tissue culture dishes or surfaces containing collagen. Many hepatic-tissue engineering approaches involve seeding isolated hepatocytes on surfaces, e.g., biodegradable polymer films,[4] three-dimensional porous polymeric scaffolds,[5–7] ligand modification polymers,[8,9] natural ECM components,[10,11] and collagen foams,[12] which have been shown to enhance hepatocyte adhesion. The cells, however, eventually de-differentiate and lose their hepato-specific function. Coordinated communication and heterotypic cell interactions are central to the function of many tissues, e.g., hepatocyte functions are enhanced when co-cultured with fibroblasts or endothelial cells[13] and blood vessels form when endothelial cells are allowed to interact with smooth muscle cells.[14] The extent of these heterotypic cell interactions is important in the development of functionally engineered tissues. To engineer liver tissues that maintain hepatic functions *in vitro* require co-cultivation of primary hepatocytes with a variety of nonparenchymal cells such as fibroblasts,[15] epithelial cells,[16] stellate cells,[17] and liver epithelial cells.[18–20] Yamato and coworkers have reported that cell attachment and detachment on/from matrix grafted with thermo-responsive polymer can be regulated by temperature.[21,22] Ito and coworkers immobilized PIPAAm in a specific pattern on a polystyrene plate, and reported that cell behavior could be controlled regiospecifically on the conjugate plate by temperature.[23,24] A limitation of traditional co-culture systems is their inability to control cell placement and manipulate cell–surface and cell–cell interactions. Micropatterning technology has been successfully applied to overcome this limitation, nevertheless, this approach requires collagen for primary hepatocytes to attach. Alternatively, designing material interfaces that possess properties that promote cellular adhesion by mimicking extracellular matrix components while also providing precise control of the cell placement would provide the advantages of micropatterning technology but without requiring adhesive proteins.

The spatial distribution of each cell type can be controlled through soft-lithography based micropatterning techniques.[25,26] Micropatterning technology has the ability to control the placement of single cells, thus allowing one to precisely manipulate cell–cell interactions. Photolithography is the conventional patterning technique of choice[13,27,28] but this lithographic technique has a number of limitations when applied to curved, nonplanar surfaces and involves multiple and cell-unfriendly processing steps to create the patterns. Other approaches, such as microfluidic channels[29] and elastomeric membranes as stencils,[30,31] are limited in the pattern geometry and size that can be achieved. An alternative strategy, microcontact printing (μCP), introduced by Whitesides and coworkers,[32,33] provides a versatile method to chemically pattern surfaces at the nanometer scale. This technique is attractive due to its high fidelity and ease of duplication. μCP uses an elastomeric stamp to print a variety of molecules with nanometer resolution and without the need for dust-free environments and harsh chemical treatments.[32–34] The success of this approach, however, is dependent upon the relative adhesiveness of the two cell types toward the substrate.

The development of new methods of fabricating thin films that provide precise control of the three dimensional (3D) topography and cell adhesion could lead to significant advances in the fields of tissue engineering and biosensors. The layer-by-layer (LbL) assembly technique, developed by Decher in 1991,[35] is a versatile and inexpensive method of constructing polymeric thin films called "Polyelectrolyte Multilayers (PEMs)", with nanometer-scale control of ionized species. PEMs are excellent candidates for tissue engineering applications due to their biocompatibility and bioinertness,[36–38] and the ability to incorporate biological molecules, such as proteins and enzymes.[39,40] We, as well as others, have demonstrated the ability of this LbL technology to readily construct complex three-dimensional architectures.[41,42] PEM surfaces have been recently developed using weak polyelectrolytes that are resistant and adherent to fibroblast attachment.[43,44] Hammond and coworkers found PEM surfaces to be cytophobic toward primary hepatocytes.[45] Here, we present an approach for organizing two types of cells on polyelectrolyte substrates through the assembly of multilayers of polyelectrolytes that are either resistant or adhesive to primary hepatocytes.

We demonstrate that patterns of primary hepatocytes and patterned cell co-cultures can be formed without the aid of adhesive proteins using the LbL deposition of the synthetic ionic polymers. We recently reported that primary hepatocytes attach and spread preferentially on sulfonated polystyrene (SPS) surfaces over Poly(diallyldimethylammonium chloride) (PDAC) surfaces.[46] In that study, we used synthetic polymers, namely PDAC and SPS, to build the multilayers, and compared the attachment and spreading of primary hepatocytes on PEM films with

either PDAC or SPS as the top most surface, to tissue culture polystyrene surfaces (TCPS). Here, we capitalized upon this differential cell attachment and spreading of primary hepatocytes on PDAC and SPS surfaces to make patterned co-cultures of primary hepatocytes and fibroblasts on the PEM surfaces. PDAC (or SPS) was patterned on the $(PDAC/SPS)_{10}$ surfaces by using a polymer-on-polymer stamping (POPS) process developed by Hammond and coworkers.[47,48] Primary hepatocytes were then seeded and preferentially attached onto the SPS surfaces. We then seeded the second cell type, fibroblasts, on the PDAC surface, resulting in patterned co-culture (Figure 1). We evaluated the cell morphology and function with an inverted microscope and biochemical assays, respectively.

Experimental Part

Materials

PDAC ($\overline{M}_w \approx 100\,000$–$200\,000$) as a 20 wt.-% solution, SPS, sodium salt ($\overline{M}_w \approx 70\,000$), fluorosilanes, and sodium chloride were purchased from Aldrich (Milwaukee, WI). Poly(dimethylsiloxane) (PDMS) from the Sylgard 184 silicone elastomer kit (Dow Corning, Midland, MI) was used to prepare stamps. The PDMS stamps were used for microcontact printing.[49] Dulbecco's Modified Eagle Medium (DMEM) with 4.5 g/l glucose, 10× DMEM, fetal bovine serum (FBS), penicillin, and streptomycin were purchased from Life Technologies (Gaithersburg, MD). Insulin and glucagon were purchased from Eli Lilly and Co. (Indianapolis, IN), epidermal growth factor from Sigma Chemical (St. Louis, MO). Purified rat albumin was purchased from Cappel Laboratories (Aurora, OH). Urea assay was purchased from Sigma Chemical. Carboxylated polystyrene latex particles (4 μm diameter) purchased from Polysciences, were used for colloidal adsorption study on patterned polyelectrolyte multilayer films. Chloromethylbenzoylaminotetramethyl rhodamine (CMTMR) and chloromethylfluorescein diacetate (CMFDA) were purchased from Molecular Probes for double immunofluorescent staining. Adult female Sprague-Dawley rats were obtained from Charles River Laboratories (Boston, MA).

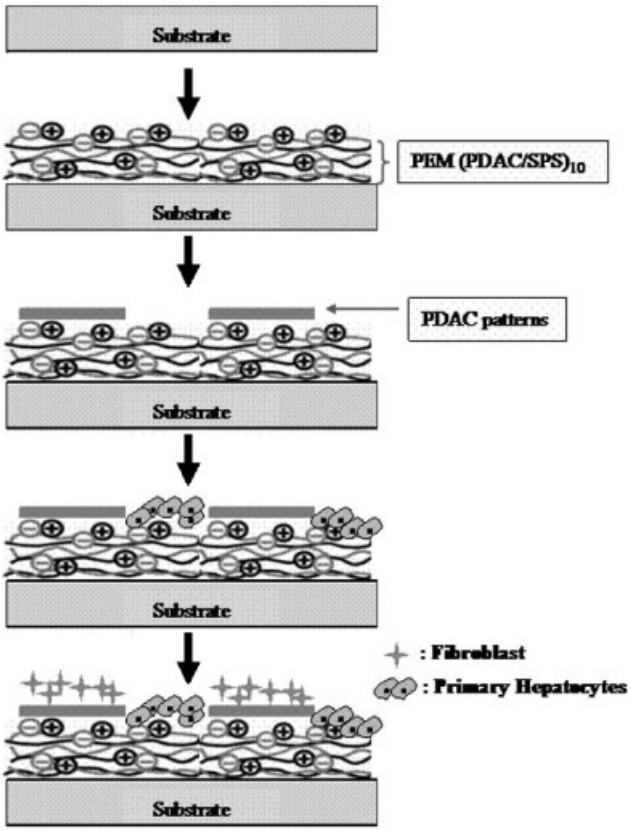

Figure 1. Schematic diagram illustrating the approach to patterning of co-cultures of primary hepatocytes and fibroblasts on PEM surfaces. PEMs $(PDAC/SPS)_{10}$ are built on top of the TCPS surface; then SPS patterns are formed using a microcontact printing technique. Primary hepatocytes are then seeded, which preferentially adhered to the SPS regions. This is followed by seeding fibroblasts onto the PDAC regions.

Preparation of PEMs

Figure 2 shows the chemical structure of the polyelectrolytes namely SPS and PDAC used to build PEM films. The PEMs were prepared as described in our earlier study.[46] Briefly, PDAC and SPS polymer solutions were prepared with deionized (DI) water at concentrations of 0.02 M and 0.01 M, respectively, (based on the repeating unit molecular weight) with the addition of 0.1 M NaCl salt. TCPS plates were subjected to a Harrick plasma cleaner (Harrick Scientific Corporation, Broading Ossining, NY) for 10 min at 0.15 Torr and 50 sccm flow of O_2 in a plasma chamber. A Carl Zeiss slide stainer equipped with a custom-designed ultrasonic bath was connected to a computer to perform LbL assembly. TCPS plates were immersed for 20 min in a polycation solution, followed by two sets of 5 min rinses with agitation. TCPS plates were subsequently placed in a polyanion solution and allowed to deposit for 20 min, followed by two sets of 5 min rinses with agitation. The samples were cleaned for 3 min in an ultrasonic cleaning bath after depositing a layer of polycation/polyanion pair. The sonication step removed weakly bounded polyelectrolytes on the substrate, forming uniform bilayers. This process was repeated to build multiple layers. All experiments were performed using ten (i.e., 20 layers) or ten and a half bilayers (i.e., 21 layers).

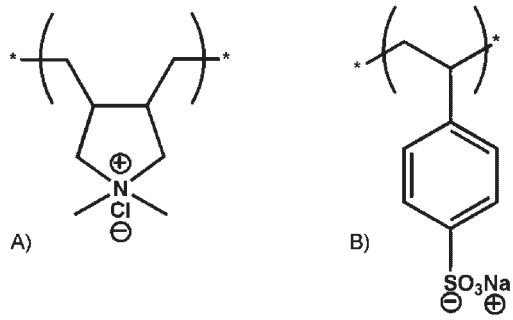

Figure 2. Chemical structure of polyelectrolytes used to build the PEMs: (A) PDAC and (B) SPS.

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DOI: 10.1002/mabi.200600205

Preparation of PDMS Stamps

PDMS stamp was made by curing the polymer on a microfabricated silicon master, which acts as a mold, to allow the surface topology of the stamp to form a negative replica of the master.[50] The PDMS stamps were made by pouring a 10:1 solution of elastomer and initiator over a prepared silicon master.[32] The silicon master was pretreated with fluorosilanes to facilitate the removal of the PDMS stamps from the silicon master. The mixture was allowed to cure overnight at 60 °C. The masters were prepared in the BioMEMS facilities at MGH East and consisted of various features (squares and lines). The polyelectrolytes were stamped onto the multilayer system using the POPS process developed by Hammond and coworkers.[48]

Cell Culture

Hepatocyte Isolation

Primary rat hepatocytes were isolated from two months old adult female Sprague-Dawley rats, according to a two-step collagenase perfusion technique described by Seglen [51] and modified by Dunn.[52] The liver isolations yielded 150–300 $\times 10^6$ hepatocytes. Using trypan blue exclusion the viability ranged from 90 to 98%. Primary hepatocyte culture medium consisted of DMEM supplemented with 10% FBS, 14 $ng \cdot ml^{-1}$ glucagon, 20 $ng \cdot ml^{-1}$ epidermal growth factor, 7.5 $\mu g \cdot ml^{-1}$ hydrocortisone, 200 $\mu g \cdot ml^{-1}$ streptomycin (10 000 $\mu g \cdot ml^{-1}$) – penicillin (10 000 $U \cdot ml^{-1}$) solution, and 0.5 $U \cdot ml^{-1}$ insulin.

Hepatocyte Culture

The cells were seeded under sterile tissue culture hoods and maintained at 37 °C in a humidified air/CO_2 incubator (90/10 vol.-%). Primary hepatocytes were cultured on PEM coated 6-well TCPS. The multilayer coated TCPS plates were sterilized by spraying with 70% ethanol and exposing them to UV light before seeding the cells onto these surfaces. The cell culture experiments were performed on PEM surfaces without adhesive proteins. Collagen coated TCPS and uncoated TCPS were used as controls in these studies. A collagen gel solution was prepared by mixing 9 parts of the 1.2 $mg \cdot ml^{-1}$ collagen suspension in 1×10^{-3} M HCl with 1 part of concentrated (10×) DMEM at 4 °C. The control wells were coated with 0.5 ml of this collagen gel solution and the coated plates were incubated at 37 °C for 1 h. Freshly isolated hepatocytes were seeded at a density of 1×10^6 cells per well on the various surfaces. 1 ml of fresh medium was supplied daily to the cultures after the removal of the supernatant. Samples were kept in a temperature and humidity controlled incubator.

NIH 3T3 Culture

NIH 3T3 fibroblast cell lines were purchased from American Tissue Type Collection. Cells grown to 70% confluency were trypsinized in 0.01% trypsin (ICN Biomedicals) solution in PBS for 10 min and resuspended in 25 ml of medium. Approximately 10% of the cells were seeded into a fresh tissue culture flask and the rest of the cells were used for the co-culture experiments. Fibroblast medium consisted of DMEM with high glucose, supplemented with 10% bovine calf serum and 200 $U \cdot ml^{-1}$ penicillin and 200 $\mu g \cdot ml^{-1}$ streptomycin.

Co-Culture on PEM Surfaces

Six-well plates were coated with PEM surfaces and rinsed in sterile water and sterilized under UV light overnight. Primary hepatocytes were seeded onto the PEM surfaces at a cell density of 1.0×10^6 per well in a serum-free medium for 36 h at 37 °C, 10% CO_2, balance air. The substrate was then rinsed three times with PBS by pipetting. On the hepatocyte-containing substrates, NIH 3T3 cells were seeded at a density of 0.5×10^6 $cells \cdot well^{-1}$ and incubated in primary hepatocyte medium at 37 °C. The fibroblast/hepatocyte ratio used in this study was 0.5:1 which is the approximate physiologic ratio of stromal:parenchymal cells in the liver.[27] The reusability of these patterns was also examined. The cells were removed from the patterns with trypsin-EDTA and washed with PBS to ensure that the cells were completely removed from the patterned surfaces. A fresh batch of primary hepatocytes was subsequently seeded onto the reused patterns. A Leica inverted phase contrast microscope with Soft RT 3.5 software was used to capture images of cell density, morphology, and spreading on the multilayer surfaces.

Cell Fluorescent Staining

The patterned co-cultures of primary hepatocytes and fibroblast were observed with a double immunofluorescent staining method. The attached primary hepatocytes were rinsed three times with 1 × PBS. The cells were incubated with 1 ml of 10×10^{-6} M CMTMR orange dye (dilution of 1:1 000 in serum free medium) for 45 min at 37 °C. The cells were then washed three times with PBS followed by medium addition. Three hours after staining the primary hepatocytes, fibroblast cells were seeded onto the stained hepatocytes. The co-cultures were washed with PBS three times and fed hepatocyte medium. Cell morphology was observed using a phase contrast and fluorescent microscope (Leica inverted microscope).

Biochemical Assays

Albumin synthesis is a widely accepted marker of hepatocyte synthetic function and urea production is an indicator of intact nitrogen metabolism and detoxification. The biochemical assays were performed on the collected supernatant. Albumin concentration was determined by an enzyme-linked immunosorbent assay, described previously, using a polyclonal antibody to rat albumin.[52] A standard curve was derived using chromatographically purified rat albumin dissolved in the medium. Urea levels were measured with commercially available kits based upon its specific reaction with diacetyl monoxime. The urea and albumin secretions were normalized to the cell number seeded on the surface (per 1×10^6 $cells \cdot d^{-1}$). Statistics was performed using the Student's *t*-test. A p value of 0.05 or lower was considered to be significant.

Determination of the Number of Cells on the Projected Area

The number of cells on the projected cell area on the different surfaces were measured using the Image J software. The projected cell area refers to the area occupied by the cells as seen under the microscope. Statistics was performed using the Student's *t*-test. A *p* value of 0.05 or lower was considered to be significant.

Results

We demonstrate that patterns of primary hepatocytes and co-cultures can be formed using the LbL deposition of ionic polymers without the aid of adhesive proteins. In this study, we used synthetic polymers, PDAC, and SPS as the polycation and polyanion, respectively, to build the PEMs. SPS patterns were formed on PEM surfaces either by microcontact printing of SPS onto PDAC surfaces or vice versa. When primary hepatocytes were seeded on top of the patterned PEM surfaces, they attached and spread predominantly on the SPS surfaces resulting in primary hepatocyte patterns. Once the hepatocytes were attached, fibroblasts were subsequently seeded and attached to the PDAC surfaces. As a result, co-culture patterns of fibroblasts and primary hepatocytes were obtained on synthetic PEM surfaces. The morphology of the cell co-cultures was characterized using phase contrast and fluorescence microscopy and their hepatic-specific functions were determined by urea and albumin synthesis.

Primary Hepatocyte Culture on PEMs

Figure 3 compares the morphology of primary hepatocytes on PEM surfaces to collagen coated TCPS control. The difference in the projected cell area for primary hepatocytes on the different surfaces is shown in Table 1. The number of primary hepatocytes that attached on to the SPS surfaces on days 1 and 5 (204 and 189 cells $\cdot$ mm^{-2}, respectively), were comparable to the number of hepatocytes that attached on to the collagen coated TCPS control surfaces on day 1 and 5 (210 and 185 cells $\cdot$ mm^{-2}), see Figure 3 and Table 1. In contrast, fewer cells attached and spread on PEM films with PDAC as the topmost surface on day 1 and day 5 (110 and 15 cells $\cdot$ mm^{-2}, respectively). Primary hepatocytes attached and spread on SPS surfaces and the morphology of the cells was comparable to the

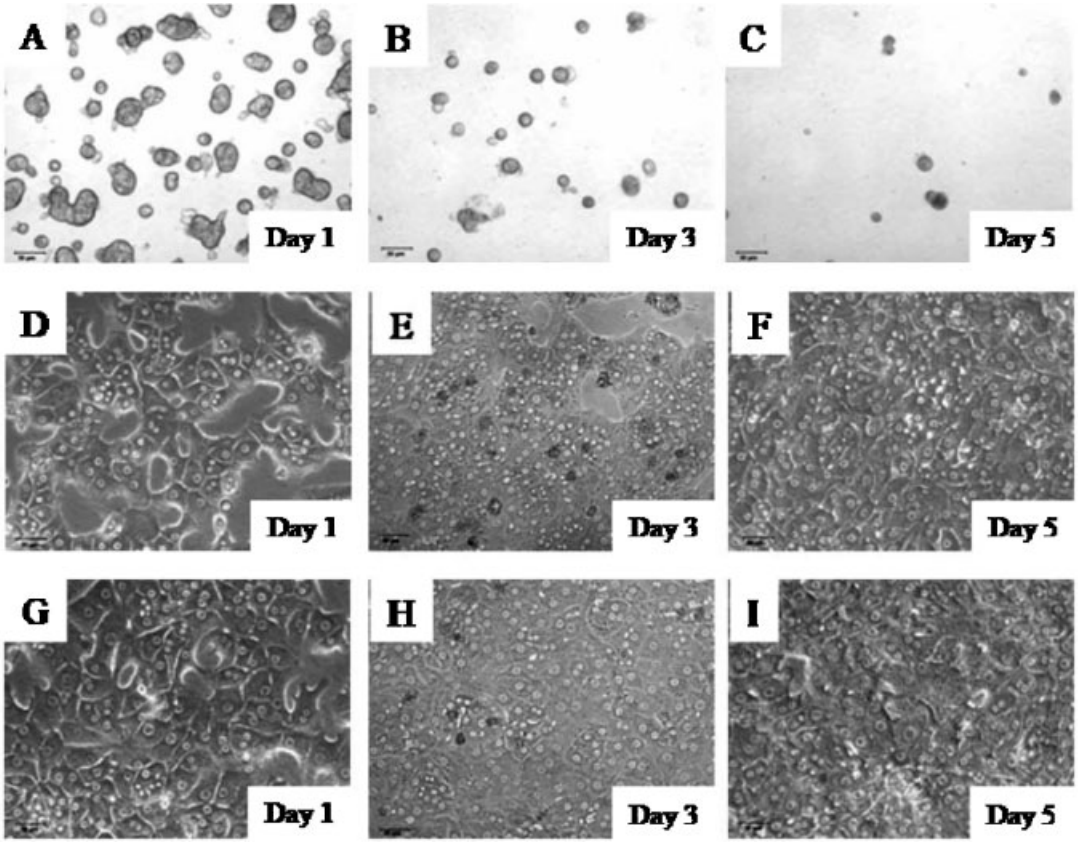

Figure 3. Phase contrast microscope images of primary hepatocyte cells seeded at 0.5×10^6 cells $\cdot$ ml^{-1} on days 1, 3, and 5 post seeding. (A–C) $(PDAC/SPS)_{10.5}$ – topmost surface PDAC, (D–F) $(PDAC/SPS)_{10}$ – topmost surface SPS, (G–I) TCPS as a control (Scale bar, 50 μm).

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DOI: 10.1002/mabi.200600205

Table 1. Primary hepatocyte cell numbers on the projected area on the different surfaces used in the study after 1, 3, and 5 d. Student's *t*-test was used for analyzing the differences between the cell adhesion on various surfaces.

Surfaces	Primary hepatocytes (4×10^5/substrate initial concentration)		
	Cells · mm^{-2}		
	After 1 d	After 3 d	After 5 d
Collagen coated TCPS control	210 ± 21	193 ± 18	185 ± 17
PDAC	110 ± 19[a)]	38 ± 8[a)]	15 ± 2[a)]
SPS	204 ± 16	192 ± 20	189 ± 21

[a)] $p < 0.05$ compared with primary hepatocyte adhesion on collagen coated TCPS control.

control. On day 1, some cells (110 cells · mm^{-2}) attached on the PDAC surface but did not spread. By day 5, most of the primary hepatocytes (15 cells · mm^{-2}) lifted off the PDAC surfaces. We further assessed the maintenance of liver-specific functions (urea and albumin secretion) over 7 d of continuous culture on these surfaces. The urea and albumin secreted by the primary hepatocytes on the PDAC surfaces decreased to zero by day 7 indicating that the primary hepatocytes do not attach on the PDAC surfaces. The urea secreted by the primary hepatocytes on the SPS surfaces decreased from day 1 [112.24 ± 2.81 μg · (10^6 cells)$^{-1}$ · d^{-1}] to day 7 [22.55 ± 1.30 μg · (10^6 cells)$^{-1}$ · d^{-1}] suggesting that the cells were de-differentiating.

Patterned Culture of Primary Hepatocytes on PEMs

Figure 4(A) illustrates the patterns used in the study. We capitalized upon the cell adhesive/resistive property of SPS and PDAC, respectively, to make patterns of primary hepatocytes. The technique of POPS makes the task of micropatterning PEMs a simpler process.[48] For POPS, a polyelectrolyte applied to a patterned stamp is transferred to a polyelectrolyte multilayer surface of the opposite charge. In our study, SPS patterns were formed on PEM surfaces either by μCP SPS onto $(PDAC/SPS)_{10.5}$ surface or μCP PDAC onto $(PDAC/SPS)_{10}$ surface using the POPS technique. We used a PDMS stamp to create square patterns of PDAC on SPS surfaces. The transfer efficiency of PDAC onto the PEM films was ascertained from optical images of negatively charged carboxylated polystyrene PS particles (diameter = 4 μm) atop the PDAC patterns. PDAC was stamped on top of the $(PDAC/SPS)_{10}$ as shown in Figure 4(B) and the colloidal particles deposited selectively onto the positive PDAC surfaces. Figure 5 illustrates the attachment of primary hepatocytes on PDAC and SPS patterns after one and five days of culture. When presented with the micropatterned surface, primary hepatocytes adhered only to the SPS regions resulting in patterns of hepatocytes. On day 1, primary hepatocytes attached preferentially on the SPS regions resulting in cell patterns irrespective of whether PDAC or SPS was stamped on top of the PEM surface [Figure 5(A) and 5(C)]. The hepatocyte patterns attached and maintained their differentiated morphology for the first few days but by day 5 began to detach from the PEM surfaces. Few hepatocytes remained attached to the patterns by day 6 [Figure 5(B) and 5(D)].We also examined the reusability of these patterns. The cells were removed from the patterns with trypsin-EDTA and washed with PBS to ensure that the cells were completely removed from the patterned surfaces. A fresh batch of primary hepatocytes was subsequently seeded onto the reused patterns. The patterns were reused four times and maintained their pattern design upon each reuse (data not shown).

Patterned Co-Cultures of Primary Hepatocytes with Fibroblasts

Co-cultures of primary hepatocytes with nonparenchymal cells such as fibroblasts have been shown to maintain hepatic functions *in vitro* for up to five weeks.[15] The fibroblast/hepatocyte ratio used in the present study is 0.5:1 which is the approximate physiologic ratio of stromal: parenchymal cells in the liver.[27] Figure 6 illustrates

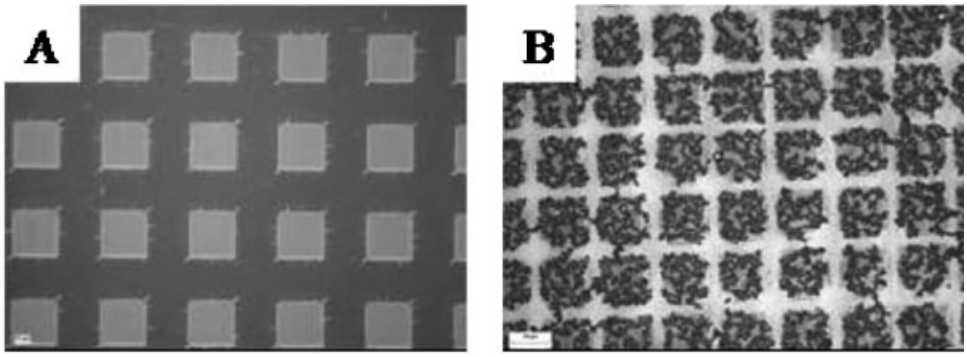

Figure 4. Optical micrographs of (A) patterns on silicon master and (B) PDAC patterns on $(PDAC/SPS)_{10}$ multilayers immersed in negatively charged carboxylated polystyrene PS particles (diameter = 4 μm). PDMS stamp with 250 μm square patterns separated by 250 μm width were used.

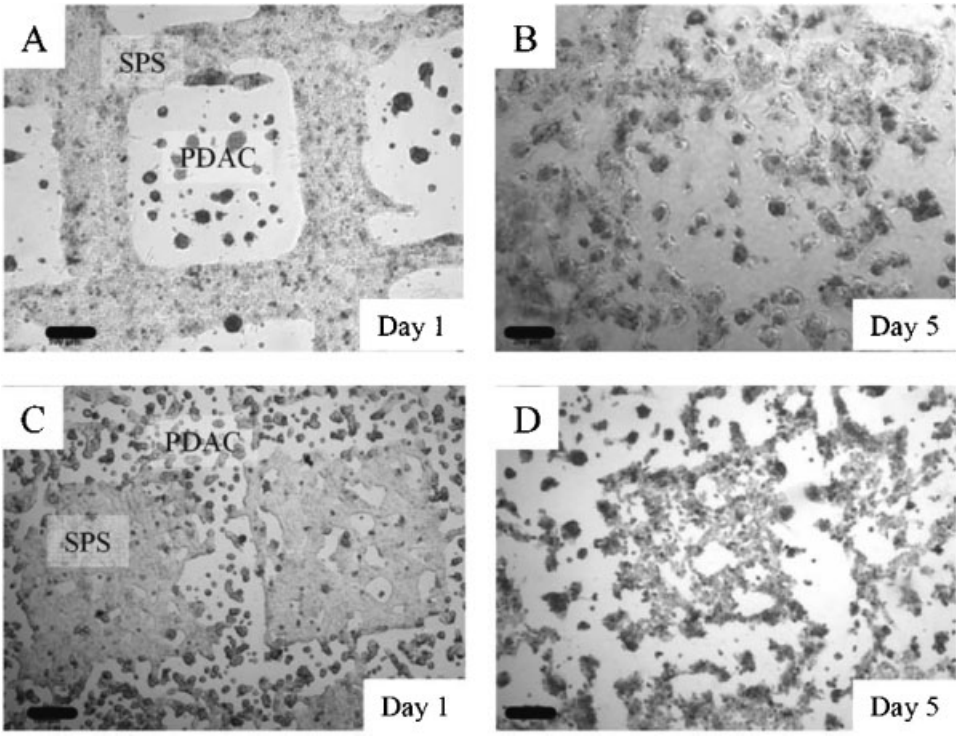

Figure 5. Phase contrast microscope images of primary hepatocyte cells seeded at 0.5×10^6 cells $\cdot$ ml^{-1} on days 1 and 5 postseeding on patterned PEM surfaces. (A), (B) Primary hepatocytes on PDAC patterns on days 1 and 5, respectively. (C), (D) Primary hepatocytes on SPS patterns on days 1 and 5, respectively. PDMS stamp with 1 000 μm square patterns separated by 250 μm width were used. (scale bar, 100 μm).

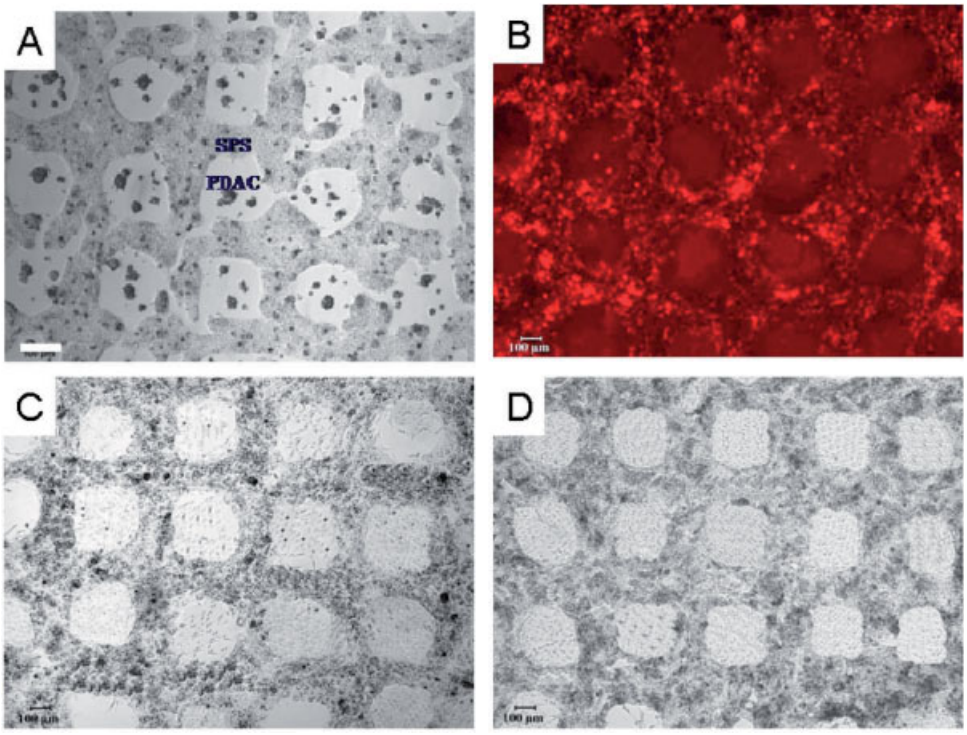

Figure 6. Patterned co-cultures of primary hepatocytes with fibroblasts. (A) Phase contrast and (B) fluorescent images of patterned primary hepatocytes (red) on PDAC patterns on day 1, phase contrast image of co-culture of primary hepatocytes with fibroblasts on (C) day 6, and (D) day 19. PDMS stamp with 250 μm square patterns separated by 250 μm width were used (scale bar, 100 μm).

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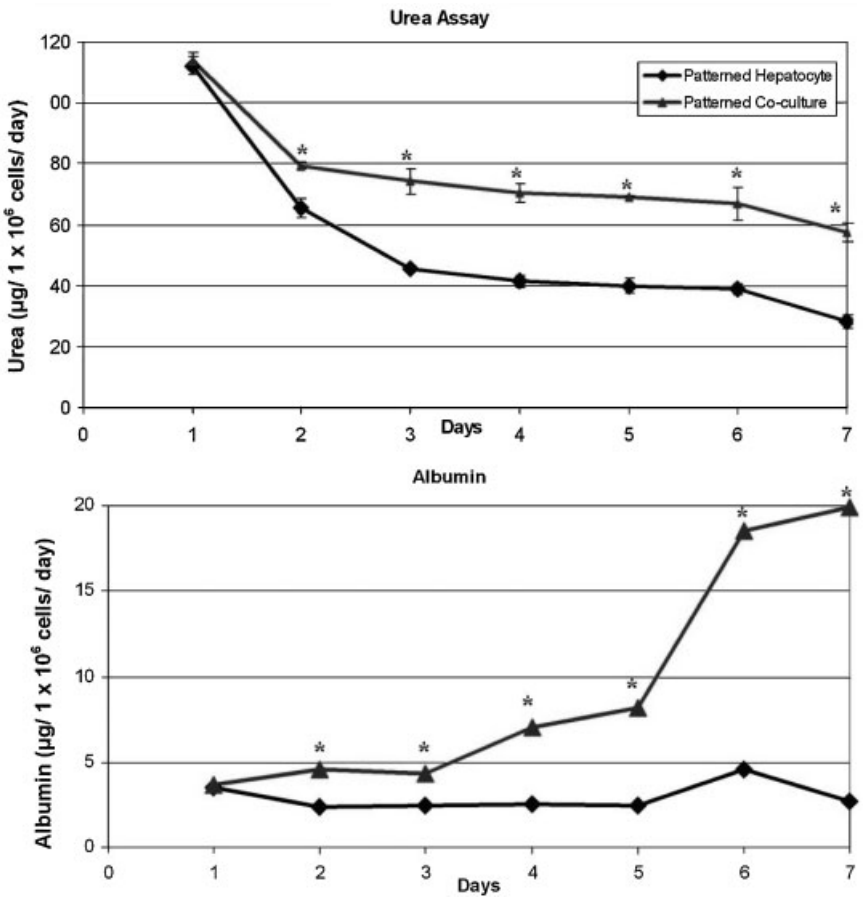

Figure 7. Liver-specific function of primary hepatocytes on PEM surfaces. (A) Urea synthesis of patterned hepatocytes and patterned co-cultures. (B) Albumin synthesis of patterned hepatocytes and patterned co-cultures ($n = 6$). Data represent mean ± S.E. of six independent experiments ($^{*}p < 0.05$ compared with patterned single hepatocyte culture).

patterned co-cultures of primary hepatocytes with fibroblast on PEM surfaces. The preferential attachment of primary hepatocytes to SPS surfaces enabled the use of this system as a template for patterned co-cultures with fibroblasts on synthetic PEM surfaces. Primary hepatocytes remained attached [Figures 6(C) and 6(D)] on the patterned co-culture system for up to 3 wk. To assess the liver-specific function, we measured the levels of urea and albumin synthesis for the patterned single culture and co-cultures for up to 7 d, as shown in Figure 7. The metabolic response of the single and co-cultures of hepatocytes on patterned PEM films were compared. Panels A and B illustrate the rate of urea and albumin production, respectively, for cultures up to one week. By day 7, liver-specific functions for the patterned co-culture [60 $\mu g \cdot (10^6 \text{ cells})^{-1} \cdot d^{-1}$ of urea and 20 $\mu g \cdot (10^6 \text{ cells})^{-1} \cdot d^{-1}$ of albumin] were much higher than the patterned single cultures [28.3 $\mu g \cdot (10^6 \text{ cells})^{-1} \cdot d^{-1}$ of urea and 2.7 $\mu g \cdot (10^6 \text{ cells})^{-1} \cdot d^{-1}$ of albumin]. The urea and albumin secreted by our patterned co-culture system was also comparable to levels secreted in the culture system developed by Bhatia et al. (80 $\mu g \cdot (10^6 \text{ cells})^{-1} \cdot d^{-1}$ of urea and 15 $\mu g \cdot (10^6 \text{ cells})^{-1} \cdot d^{-1}$ of albumin)[27] for a similar fibroblast and hepatocyte culture ratio (0.5:1), although different pattern sizes and shapes were used in these two studies.

Discussions

In this study, we demonstrated an alternative approach to engineer patterned cell co-culture of primary hepatocytes and fibroblast without the aid of adhesive proteins using the PEM films. In Table 2, we compared the maximum achievable levels of an hepatic-specific function in our co-culture system to *in vivo* and other *in vitro* hepatocyte co-culture systems studied, namely, the collagen double gel and the co-culture system studied by Bhatia and coworkers. By comparison, the human liver *in vivo,* consisting of 150–250 $\times 10^9$ hepatocytes, secretes approximately 5–8 $\mu g \cdot (10^6 \text{cells})^{-1} \cdot h^{-1}$ of urea and 2–3.3 $\mu g \cdot (10^6 \text{cells})^{-1} \cdot h^{-1}$ of albumin.[53] The hepatic-specific function obtained on the PEM films was comparable to the collagen coated tissue-culture polystyrene (TCPS) surfaces, the collagen double gel, the co-culture system, and the *in vivo* human liver. Primary hepatocytes cultures using previously well established techniques are stable, but there are certain disadvantages associated with each of these methods.

Advantages of Our Culture System Over Other Hepatocyte Culture Systems

Collagen sandwich (double gel) cultures preclude direct cell–cell interaction between the sandwiched hepatocytes and other cells types that may be cultured atop the collagen sandwich. Donato et al. used transwells to form co-cultures, therefore the cells were not in direct contact with each other.[54] This culture system imposed an artificial boundary that precluded cell–cell interactions. Shimaoka et al. developed a method whereby hepatocytes were cultured onto cover slips and the cover slips were subsequently added to the center of a confluent culture of

Table 2. Comparison of maximum achievable levels of hepatic-specific function (urea and albumin secreted) in various hepatocyte culture systems. Urea and albumin secreted per 1×10^6 cells were approximated from experimental data and available literature.

Hepatocyte culture systems	Urea secreted	Albumin secreted
	$\mu g \cdot (10^6 cells)^{-1} \cdot h^{-1}$	$\mu g \cdot (10^6 cells)^{-1} \cdot h^{-1}$
Human liver *in vivo*[a)]	5–8	2–3.3
Single collagen gel[b)]	1.2–2.0	0.1–0.3
Sandwich gel[c)]	3–4	1–2
Random co-culture of hepatocytes and fibroblast in 0.5:1 ratio[d)]	2–3.5	0.25–0.4
Patterned co-culture of hepatocytes/fibroblast in 0.5:1 ratio[d)]	4–5	1–2
Our hepatocytes/fibroblast co-culture system in 0.5:1 ratio[c)]	11.8 ± 1.1	2.2 ± 0.1

[a)]From ref.[53]; [b)]From ref.[52]; [c)]From our experiments; [d)]From ref.[2,27].

fibroblast.[55] This method resulted in significant cell death underneath the cover slip. Furthermore, significant topological variations in the culture existed which caused variations in the degree of cell–cell interactions. Bhatia et al. developed a patterned co-culture system using photolithography.[27] This method was very effective in controlling cell contact and adhesion, but the lithographic technique used has a number of limitations when applied to curved, nonplanar surfaces and involved multiple and cell-unfriendly processing steps to create the patterns. Furthermore, collagen must be added to the patterns in order for the primary hepatocytes to adhere onto the surface.

In the present study we used μCP which has several advantages over the method used by Bhatia et al. The advantages include its high fidelity, ease of duplication, ability to print a variety of molecules with nanometer resolution, and without the need for dust-free environments and harsh chemical treatments.[32–34] In addition, we were able to achieve primary hepatocytes adhesion without the need of collagen or other adhesive proteins.

Conclusion

In conclusion, the present work outlined a method for controlling cell-surface interactions using polyions and PEMs. PEMs were used to produce defined cell-resistant and cell-adhesive properties depending on the topmost surface and the type of cells. We demonstrated using both the biochemical studies and the direct microscopy images of live cells that primary hepatocytes attached, spread, and maintained function on the PEM films. We also demonstrated that the LbL deposition and μCP of ionic polymers can be used as a template for patterned co-cultures of primary hepatocytes and fibroblasts. The patterned co-cultures of primary hepatocytes and fibroblasts maintained hepato-specific function much longer than the patterned single culture of primary hepatocytes. Finally, PEM films permit precise control of the 3D topography at the micro and nanometer scales,[41] thus providing an alternative and flexible tool for fabricating cell-specific surfaces for tissue engineering applications.

Acknowledgements: This work was funded by the *MSU Foundation, Michigan Economic Development Corporation*, and in part by *NSF* (BES No. 0222747, No. 0331297, and No. 0425821, and CTS No. 0609164), the *Whitaker Foundation* and the *Environmental Protection Agency*. The authors of this paper would like to thank Deanna Thompson at the Shriners Hospital for Children in Boston and Jeremy Walraven from MSU for their help in making the silicon master used in this investigation and the NIH BioMEMs Resource Center (EB002503) at Massachusetts General Hospital (MGH).

Received: September 16, 2006; Revised: November 30, 2006; Accepted: December 4, 2006; DOI: 10.1002/mabi.200600205

Keywords: adhesion; co-culture; micro-contact printing; polyelectrolyte; polyelectrolyte multilayers; primary hepatocytes; tissue engineering

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