Selective Primary Hepatocyte Adhesion on Polyelectrolyte Multilayer : Template for Patterned Cell Co-Culture

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Introduction

The ability of surfaces to modulate cellular behavior such as adhesion, migration, proliferation and differentiation is an important facet of tissue and organ formation and in the eventual realization of functional biomaterials. The presence of micro or nanostructures on a surface permits the manipulation of cell-substrate and cell-cell interactions to better control cellular function and behavior. Commonly, proteins and cells indiscriminately attach onto medically implantable surfaces, which may ultimately lead to undesired fibrous encapsulation, detrimental clinical complications, increased risk of infection, and poor device performance. [1, 2] Consequently, by generating so-called bioinert materials, one may attempt to first reduce any nonspecific physiological responses and then create a truly bioactive system by reintroducing the attachment of only desired cells in a predictable fashion by using cell specific signaling molecules or adhesion ligands,[3] often presented in precise engineered geometries.

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 ionic layer-by-layer (LBL) assembly technique, introduced by Decher in 1991,[4] has emerged as a versatile and inexpensive method of constructing polymeric thin films, with nanometer-scale control of ionized species. Films are formed by electrostatic interactions between oppositely charged poly-ion species to create alternating layers of sequentially adsorbed poly-ions are called "Polyelectrolyte Multilayers (PEMs)". Such an approach offers unprecedented nanoscale control over the film architecture and properties, including film thickness, composition, conformation, degree of interchain ionic bonding, roughness, and wettability.[5] Concomitantly, the resulting films can conformably coat substrate materials of any type, size, or shape (including implants with complex geometries and textures, e.g., stents and crimped blood vessel prostheses). Here we suggest an alternative approach, patterning with synthetic compounds, that provides flexibility for building complex 3D architectures as illustrated previously[6] and could lead to significant advances in the fields of tissue engineering.[7]

This work describes the successful attachment and spreading of primary hepatocytes on polyelectrolyte multilayer (PEM) films without the use of adhesive proteins such as collagen or fibronectin and patterns of co-cultures. We demonstrate that patterns of primary hepatocytes and co-cultures can be formed using this layer-by-layer deposition of ionic polymers. In our study, we used synthetic polymers namely poly(diallyldimethylammoniumchloride) (PDAC) and sulfonated poly(styrene) (SPS) as the polycation and polyanion, respectively, to build the multilayers. Primary

hepatocytes attached and spread preferentially on the SPS surfaces over the PDAC surfaces. SPS patterns were formed on PEM surfaces either by microcontact printing SPS onto PDAC surfaces or vice-versa. Primary hepatocytes adhered and spread only on SPS surfaces whereas fibroblasts readily attached to a variety of surfaces including both PDAC and SPS. As a result, co-culture patterns of fibroblast and primary hepatocytes were obtained on synthetic PEM surfaces without using adhesive proteins. This technique may be a useful tool for fabricating controlled co-cultures with specified cell-cell and cell-surface interactions, thus providing flexibility in designing cell-specific surfaces for tissue engineering applications.

Experimental

Materials. Poly(diallyldimethylammoniumchloride) (PDAC) (Mw~100,000-200,000) as a 20 wt % solution, sulfonated poly(styrene), sodium salt (SPS) (Mw~70,000), fluorosilanes and sodium chloride were purchased from Aldrich (Milwaukee, WI). All polymers were used without further purification. Poly(dimethylsiloxane) (PDMS) from the Sylgard 184 silicone elastomer kit (Dow Corning, Midland, MI) was used to prepare the stamps. The PDMS stamps were used for microcontact printing (μCP).[8] Dulbecco's Modified Eagle Medium (DMEM) with 4.5 g/l glucose, 10X 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 (St. Louis, MO).

Preparation of PEMs. PDAC and SPS polymer solutions were prepared with deionized (DI) water at concentrations of 0.02M and 0.01M respectively, (based on the repeating unit molecular weight) with the addition of 0.1M NaCl salt. Polyelectrolyte dipping solutions were prepared with DI water supplied by a Barnstead Nanopure-UV 4 stage purifier (Barnstead International Dubuque, Iowa), equipped with a UV source and final 0.2 μm filter. Solutions were filtered with a 0.45 μm Acrodisc syringe filter (Pall Corporation) to remove particulates. The tissue culture polystyrene surfaces (TCPS) 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. The layer-by-layer process was carried out in an automatic dipping machine (HMS programmable slide stainer from Zeiss Inc.). To form the first bilayer, the TCPS were immersed for 20 min in a polycation solution. Following two sets of 5 min rinses with agitation, the TCPS were subsequently placed in a poylanion solution and allowed to deposit for 20 min. Afterwards, the 6 well plates were rinsed twice for 5 min each. 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 half bilayers (i.e., 21 layers).

Preparation of PDMS Stamps. An elastomeric stamp was made by curing PDMS 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.[9] The PDMS stamps

were made by pouring a 10:1 solution of elastomer and initiator over a prepared silicon master.[10] 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 polymer-on-polymer stamping (POPS) process developed by Hammond and co-workers.[11]

Hepatocyte Culture System. Primary rat hepatocytes were isolated from adult female Sprague-Dawley rats (Charles River Laboratories, Boston, MA) weighing 200-300 g, according to a two-step collagenase perfusion technique described by Seglen[12] and modified by Dunn.[13] Hepatocytes were cultured on PEM coated 6-well tissue culture polystyrene surfaces (TCPS). All the multilayer coated TCPS were sterilized by spraying with 70 % ethanol and exposing them to UV light before culturing the cells onto these surfaces. The cell culture experiments on the PEM surfaces were performed without coating the surfaces with any adhesive proteins. Collagen coated TCPS and uncoated TCPS were used as controls in these studies. The collagen gelling solution was prepared by mixing 9 parts of the 1.2 mg/ml collagen suspension in 1 mM HCl with 1 part of concentrated (10X) DMEM at 4°C. The control wells were coated with 0.5 ml of this collagen gelling solution and the coated plates were incubated at 37°C for 1 hour. Freshly isolated hepatocytes were seeded at a density of 4×10^5 cells per well for 7 days. The standard hepatocyte culture medium consisted of DMEM supplemented with 10% FBS, 14 ng/ml glucagon, 20 ng/ml epidermal growth factor, 7.5 µg/ml hydrocortisone, 200 µg/ml streptomycin (10,000 µg/ml) – penicillin (10,000 U/ml) solution, and 0.5 U/ml insulin. One ml of fresh medium was supplied daily to the cultures after removal of the supernatant. Samples were kept in the incubator where the temperature and humidity were properly controlled. 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 Culture on PEM Surfaces PEM surfaces were rinsed in sterile water and sterilized under UV light overnite. Hepatocytes were seeded onto the PEM surfaces at a cell density of 0.4×10^6/substrate in a serum-free media for 18 h at 37°C, 10% CO_2, balance air. The substrate was then rinsed three times with PBS by pipetting. NIH 3T3 fibroblast cell lines were purchased from American Tissue Type Collection. Cells grown to 70% confluence were trypsinized in 0.01% trypsin (ICN Biomedicals) solution in PBS for 10 min and re-suspended in 25mL media. Approximately 10% of the cells were seeded into a fresh tissue culture flask. Culture medium consisted of DMEM with high glucose, supplemented with 10% bovine calf serum and 200U/mL penicillin and 200µg/mL streptomycin. On the hepatocyte-adhered substrates, NIH 3T3 cells (0.2×10^6 cells/ml) were seeded and then incubated in a DMEM containing FBS (2 ml) at 37°C. A Leica inverted phase contrast microscope was used to capture images of cell density, morphology, and spreading on the multilayer surfaces.

Results and Discussion

Controlling the attachment of fibroblasts and primary hepatocytes presented a set of interesting challenges. Fibroblasts are robust cells that adhere indiscriminately to

a variety of surfaces by attaching to their own secreted ECM proteins. On the other hand, primary hepatocytes exhibit more selective behavior *in vitro*, preferentially attaching on surfaces containing collagen.[14]

Primary hepatocytes attached and spread on PEM films with SPS as the topmost surface. In contrast, fewer cells attached and spread on PEM films with PDAC as the topmost surface**.** As seen in Figure 1, the primary hepatocytes seeded onto multilayers composed of SPS and PDAC show varying affinity on the three surfaces when compared to the TCPS controls. The cells on $(PDAC/SPS)_{10.5}$ with PDAC as the topmost surface show essentially no attachment to these surfaces but rather simply float in the cell culture media while the $(SPS/PDAC)_{10}$ films with SPS as the topmost surface allow cell attachment comparable to the control. The interesting aspect of this result is that the primary hepatocytes attached to the artificial SPS surface without the help of collagen and did not require attachment of ligands or proteins.

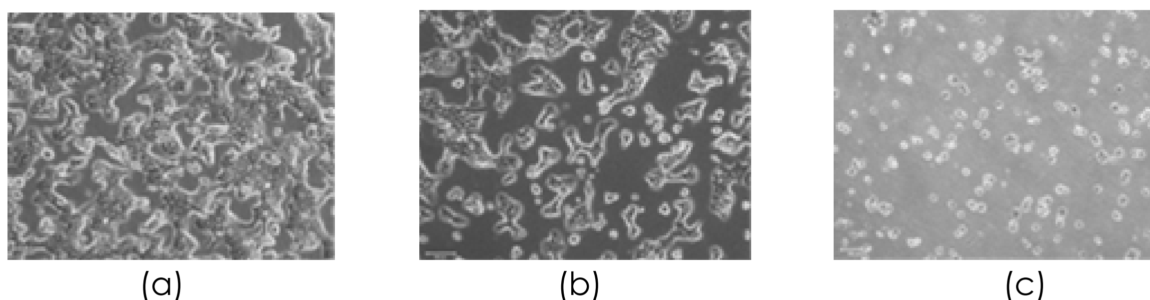

Figure 1. *Phase contrast microscope images taken of primary hepatocyte cells seeded at 0.5x10^{6} cells/ml on day 2 post seeding; (a) tissue culture grade polystryrene (TCPS) as a control (b)* $(SPS/PDAC)_{10}$ *(c)* $(PDAC/SPS)_{10.5}$ *(Scale bar, 100 μm)*

One of the major challenges in studying the mechanism of cell-substrate interaction on synthetic surfaces is discerning the relative role of the chemical functional groups on this interaction. Therefore, we evaluated several synthetic sulfonic acid polymers with distinct chemical structures and molecular mass for this purpose.[15] The PAS polymer has a similar structure to SPS but contains a hydrophobic ether group in the benzene ring while the PVS polymer has no benzene ring. These polymers were chosen to determine the functional group responsible for the observed cellullar behavior on the PEM surfaces. Primary hepatocytes attached and spread on PEM films with all three sulfonic acid polymers as the topmost surface. The similarity in the results suggests that the sulfonate group was likely responsible for the primary hepatocyte attachment and spreading on the PEM surface. The morphology observed on SPS and other sulfonate surfaces were consistent with cells demonstrating affinity towards the surface. Similar behavior was not observed when hepatocytes were cultured on PDAC surfaces. Primary hepatocytes were also grown on various positive surfaces such as LPEI and BPEI to observe the importance of charge effect on cell adhesion and spreading. Primary hepatocytes attached and spread on $(LPEI/SPS)_{10.5}$ and $(BPEI/SPS)_{10.5}$ suggesting that charge effect was not likely the mechanism for cell adhesion.

Conclusion

In conclusion, the present work outlines a method for controlling cell-surface interactions using various polyions in building the PEMs. PEMs were used to produce defined cell-resistant and cell-adhesive properties depending on the topmost surface and the type of cells used. We have shown using both biochemical studies and direct microscopy imaging of live cells that primary hepatocytes attach, spread and function on PEM films without the aid of adhesive proteins. These results demonstrate the feasibility of attaching primary hepatocyte directly on PEMs. We also demonstrated that patterns of primary hepatocytes can be formed using this layer-by-layer deposition of ionic polymers, which can be used as a template for patterned cell co-cultures. Further, we cultures.

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