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Controlling Primary Hepatocyte Adhesion and Spreading on Protein-Free Polyelectrolyte Multilayer Films

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This Communication 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. We demonstrate for the first time that *primary hepatocytes* attached, spread, and maintained differentiated function, such as albumin and urea production, on a synthetic PEM surface with poly(4-styrenesulfonic acid) (SPS) as the topmost surface. Albumin synthesis is a widely accepted marker of hepatocyte synthetic function, and urea production is an indicator of intact nitrogen metabolism and detoxification. Extracellular matrix (ECM) proteins such as collagen or related peptides are commonly used in preparing thin films for making cell patterns either by self-assembled monolayers or "soft lithography".[1] Here we suggest an alternative approach, patterning with synthetic compounds, that provides flexibility for building complex three-dimensional (3D) architectures as illustrated previously[2] and could lead to significant advances in the fields of tissue engineering.[3]

The ionic layer-by-layer (LbL) assembly technique, introduced by Decher in 1991,[4] forms films by electrostatic interactions between oppositely charged poly-ion species. The alternating layers of sequentially adsorbed poly-ions are called "polyelectrolyte multilayers". PEMs have become excellent candidates for biomaterial applications due to (1) their biocompatibility and bioinertness,[5−7] (2) their ability to incorporate biological molecules, such as proteins,[8,9] and (3) the high degree of molecular control of the film structure and thickness, providing a much simpler approach to construct complex 3D surfaces as compared with photolithography.[10] We, as well as others, have demonstrated the ability of this layer-by-layer technology to readily construct complex 3D surfaces.[2,11] The biodegradability of PEMs has been demonstrated by Lynn and co-workers.[12] Thus far, the suitability of these films for biomedical applications has been illustrated predominantly with immortalized cell lines, such as fibroblast, osteoblast, and endothelial cells.[5] Rubner and co-workers have recently developed PEM surfaces using weak polyelectrolyte systems that were cytophobic and cytophilic to fibroblast attachment.[7]

Fibroblasts can attach to many surfaces due to their robustness and ability to secrete their own extra-cellular matrix proteins and thus provide a mechanism by which they can promote their own attachment to surfaces. On the other hand, *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.[13] Primary hepatocytes are anchorage dependent and must attach to maintain their differentiated function. A stationary suspension culture of isolated hepatocytes typically loses its differentiated function within hours. Primary hepatocytes maintain their viability in tissue culture for up to 2 weeks; nevertheless, they lose their differentiated function within a few days. To address this limitation, approaches such as overlaying the hepatocytes with a collagen gel or using cylindrical collagen gel entrapment have extended the differentiated functions in vitro for at least 5−6 weeks.[13,14] Likewise, co-cultures with nonparenchymal cells have extended the differentiated function of primary hepatocytes in vitro.[15−17] Here we report culturing primary hepatocytes on a collagen-free synthetic SPS surface; these cultured cells maintain a level of differentiated function similar to that of a tissue culture polystyrene surface (TCPS). The advantage of PEM surfaces is their ability to construct three-dimensional structures with controlled cell−cell and cell−surface interactions.

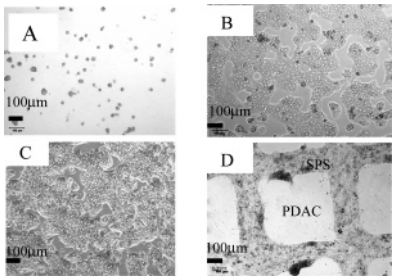

Figure 1. Optical micrographs of primary rat hepatocytes after 3 days in culture on PEM surfaces: (A) $(PDAC/SPS)_{10.5}$. (B) $(PDAC/SPS)_{10}$, (C) TCPS−control, (D) PDAC patterns on SPS. The magnification is 10×.

The aim of this study is to characterize the attachment, spreading, and function of primary rat hepatocytes cultured on PEM surfaces. To investigate the long-term effects of PEM films on cell viability and function, we assessed the morphology and maintenance of liver-specific functions over 7 days of continuous culture. In our study, we used synthetic polymers, namely poly(diallyldimethylammonium chloride) (PDAC) and SPS, as the polycation and polyanion, respectively, to build the multilayers. We compared the attachment and spreading of primary hepatocytes on PEM films, with either PDAC or SPS as the topmost surface, to TCPS surfaces with and without collagen, as the control. The PEM surfaces used for the cell adhesion studies were not coated with collagen or other adhesive proteins.

Figure 1 compares the morphology of primary hepatocytes on PEM surfaces with collagen-coated TCPS obtained with phase contrast microscopy. Primary hepatocytes attached and spread on PEM films with SPS as the topmost surface. In contrast, the attached cells did not spread on PEM films with PDAC as the topmost surface and eventually lifted off the surface.[18] We capitalized upon this cell adhesive/resistive property of SPS and PDAC, respectively, to make patterns of primary hepatocytes. SPS patterns were formed on PEM surfaces either by microcontact printing SPS onto PDAC surfaces or vice versa using the polymer-on-polymer stamping technique developed by Hammond and co-workers.[10] Primary hepatocytes adhered and spread only on SPS surfaces, resulting in primary hepatocyte cell patterns (Figure 1D), whereas fibroblasts readily attached to a variety of surfaces, including both PDAC and SPS.

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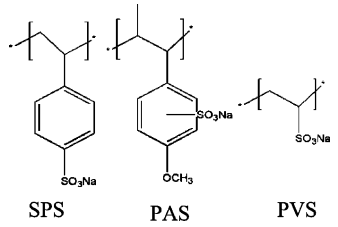

Figure 2. Structural formulas of the sulfonic acid polymers.

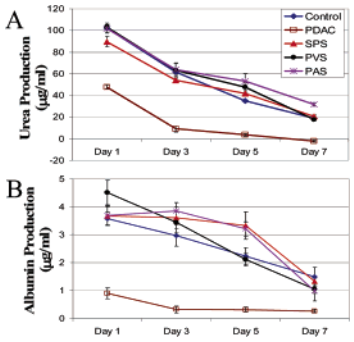

Figure 3. Metabolic function of primary hepatocytes on PEM surfaces. (A) Total urea production per day. (B) Total albumin secretion per day ($n = 6$).

This enabled the use of this system as a template for patterned co-culture of fibroblast and primary hepatocytes on synthetic PEM surfaces without adhesive proteins (data not shown).

One of the major challenges in studying the mechanism of cell−substrate interactions 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. Figure 2 shows the chemical structure of three different sulfonic acid polymers, namely SPS, poly(anetholesulfonic acid) (PAS), and poly(vinylsulfonic acid) (PVS), used to build PEM films for the primary hepatocytes studies. The PAS polymer has a structure similar to that of 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 cellular 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 was consistent with cells demonstrating affinity toward 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.[18]

The long-term metabolic response of continuous hepatocyte culture on PEM films was compared with collagen-coated surfaces, as shown in Figure 3. Panels A and B illustrate the rate of albumin and urea production, respectively, for cultures up to one week. The daily production of both albumin and urea on PEM surfaces was comparable to that of cells cultured on collagen-coated tissue culture dish. By day 7, the liver-specific functions approach zero for PEM films with PDAC as the topmost surface. This is likely due to the fact that the cells were unable to maintain attachment to the PDAC surfaces and had completely lifted from the surface by day 7. In contrast, PEM films with the sulfonated groups as the topmost surface had urea and albumin production comparable to that on the collagen-coated TCPS surface.

In conclusion, the present work outlines a method for controlling cell−surface interactions by 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, PEM films permit precise control of the three-dimensional topography at micro- and nanometer scales. Taken together, 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.

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Supporting Information Available: Procedures for preparing polyelectrolyte multilayers, cell culture, and metabolic studies on the PEM surfaces. This information is available free of charge via the Internet at http://pubs.acs.org.

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