Micropatterning Neuronal Cells on Polyelectrolyte Multilayers

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This paper describes an approach to adhere retinal cells on micropatterned polyelectrolyte multilayer (PEM) lines adsorbed on poly(dimethylsiloxane) (PDMS) surfaces using microfluidic networks. PEMs were patterned on flat, oxidized PDMS surfaces by sequentially flowing polyions through a microchannel network that was placed in contact with the PDMS surface. Polyethyleneimine (PEI) and poly(allylamine hydrochloride) (PAH) were the polyions used as the top layer cellular adhesion material. The microfluidic network was lifted off after the patterning was completed and retinal cells were seeded on the PEM/PDMS surfaces. The traditional practice of using blocking agents to prevent the adhesion of cells on unpatterned areas was avoided by allowing the PDMS surface to return to its uncharged state after the patterning was completed. The adhesion of rat retinal cells on the patterned PEMs was observed 5 h after seeding. Cell viability and morphology on the patterned PEMs were assayed. These materials proved to be nontoxic to the cells used in this study regardless of the number of stacked PEM layers. Phalloidin staining of the cytoskeleton revealed no apparent morphological differences in retinal cells compared with those plated on polystyrene or the larger regions of PEI and PAH; however, cells were relatively more elongated when cultured on the PEM lines. Cell-to-cell communication between cells on adjacent PEM lines was observed as interconnecting tubes containing actin that were a few hundred nanometers in diameter and up to 55 μm in length. This approach provides a simple, fast, and inexpensive method of patterning cells onto micrometer-scale features.

Introduction

The attachment of cells on defined surface areas (cell patterning) is an important step toward the development of devices targeted for applications in biosensors[1,2] and bioelectronics,[3] tissue engineering,[4,5] and cell biology.[6,7] The most common approach to cellular attachment involves the adsorption of extracellular proteins or other biocompatible materials onto surfaces which provide a framework for the subsequent adhesion of cells. A variety of methods used for patterning extracellular proteins and cells includes the use of self-assembled monolayers (SAM) on silicon[8] or gold,[9] photolithographic patterning of siloxanes with selective functional groups,[10] microcontact printing,[11] patterning through holes in elastomeric membranes,[12] and patterning using three-dimensional microfluidic systems to obtain alternate cell-type patterns.[13]

The majority of the methods mentioned above employ adhesive proteins to bind the cells to the surface and are limited by the susceptibility of the bonds to protein degradation. The use of nonbiological polymers as adhesive elements, however, would reduce the susceptibility to degradation and lead to the greater stability of patterned cellular regions. In this work, we present the use of polyelectrolyte multilayers (PEMs) as an adhesion layer for cell micropatterning. The PEM coatings consist of alternating layers of polyanions and polycations adsorbed onto a charged surface. The sequential deposition of these layers is based on the electrostatic attraction between the opposite charges of alternate polyions. Such multilayer assemblies are attractive for a variety of sensing, separation, and electronics applications due to their ease of synthesis and film stability. The use of PEMs to alter the functionality of a surface-charged region has already been exploited to control the flow direction and electroosmotic mobilities within plastic microfluidic systems.[14,15]

Recently, the use of PEMs has shown promise as a biocompatible coating material. The adhesion and viability

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of bone cells was observed on a number of polyelectrolytes including poly(sodium 4-styrenesulfonate) (PSS), poly-(allylamine hydrochloride) (PAH), and poly(L-lysine).[16] Also, the adhesion of chondrosarcoma cells was observed preferentially onto a polycation.[17] In more recent work, attachment of cells on a natural polyelectrolyte sheet was achieved by selectively using a blocking agent to prevent cell adhesion in certain areas but not in others.[18] Poly-electrolyte multilayers have also been used as cell-resistant surfaces on tissue-culture plates.[19] Multilayers of poly(acrylic acid) (PAA)/polyacrylamide (PAAm) and poly(methacrylic acid) (PMA)/PAA proved to be effective and stable as cell-resistant coatings for mammalian fibroblasts. Stamping a weak polycation on these cell-resistant polyelectrolytes has recently been used to pattern different RGD densities, allowing cell behavior studies at a variety of ligand densities under geometric constraints.[20] RGD is a known cell adhesion molecule, comprised of the amino acids Arg-Gly-Asp, that is found in the extracellular matrix protein fibronectin.[21] Here, we report an approach for the selective adhesion of rat retinal R28 cells on micropatterned PEM lines that were deposited onto poly-(dimethylsiloxane) (PDMS) surfaces using microfluidic networks. In this work, the PDMS is used as a tunable material by negatively charging the surface (oxidation) to promote PEM deposition in a patterned fashion and later exposing the surface to air to tune the uncoated portion of the PDMS surface back to its neutral state. By using PDMS as the tissue-culture surface, the need for further coverage of the nonpatterned surfaces is avoided since PDMS acts like a cell-resistant surface when allowed to return to its hydrophobic (neutral) condition. This simpli-fies the entire patterning process since further steps to place blocking agents, commonly required to avoid cell adhesion on undesired areas, are not needed.

Genetically engineered retinal cell lines, such as the R28 cell line, are used for studies requiring large numbers of cells derived from the retina and offer a great deal of flexibility in experimental design over retinal tissue. Retinal cell culture has proven to be a powerful tool in ophthalmic research, providing controllable experimental systems for the examination of fundamental retinal processes. The major characteristics of retinal culture systems are an indefinite lifespan, freezer storage, phe-notypic marker expression of different types of retinal cells, mitotic growth, and lack of tumor-growing ability. These cells have been shown to express markers of photoreceptor, Müller, and ganglion cell phenotypes and are useful for retinal transplantation experiments, studies on retinal gene expression, apoptosis, cytotoxicity, and neuroprotection useful in in vitro toxicology, among others studies.

Cell attachment to PEM patterns was observed within 5 h after the cells were seeded, a time frame that is comparable to the attachment observed using standard tissue-culture material (polystyrene). Excessive growth on the PEM pattern occurred after several days in culture and extended beyond the pattern onto the PDMS. As little as one polyelectrolyte layer could be used to pattern the cells successfully. This technique provides a measure of flexibility as one or more layers of the polyions can be used to pattern the cells, and it overall presents a simple, fast, and inexpensive form of patterning cells onto micrometer-scale lines.

Experimental Section

Materials. Poly(allylamine hydrochloride) (PAH, $M_W \approx$ 70 000) was purchased from Sigma-Aldrich (St. Louis, MO).[22] Poly(styrenesulfonic acid) (PSS, M_W = 70 000) and polyethyl-eneimine (PEI, M_W = 70 000) were purchased from Polysciences, Inc. (Warrington, PA). Poly(dimethylsiloxane) (PDMS, Sylgard 184) was purchased from Dow Corning (Midland, MI). Silicon wafers were obtained from Nova Electronic Materials, Ltd. (Carrollton, TX). Dubelco's Modification of Eagle's Medium/Ham's F-12 (DMEM/F12) and media supplements (penicillin–strep-tomycin, L-glutamine, MEM nonessential amino acids, and sodium pyruvate), Dulbecco's phosphate-buffered saline (DPBS), phosphate-buffered saline (PBS), and trypsin ethylenediamine-tetraacetic acid (EDTA) were obtained from Mediatech, Inc. (Hernon, VA). Fetal bovine serum was purchased from Invitrogen Corporation (Carlsbad, CA). Alexa Fluor 568 phalloidin, 4′,6-diamidino-2-phenylindole (DAPI) and 6-carboxy-2′,7′- dichlo-rodihydrofluorescein diacetate (carboxy DCFDA) were purchased from Molecular Probes (Eugene, OR).

Fabrication of the Silicon Master and PDMS Structures. Silicon masters with raised features (parallel lines, 50 μm in width) for molding PDMS microchannels were fabricated using the procedure described by Martynova et al.[23] PDMS microfluidic structures were molded by pouring the polymer on the silicon master and curing at 65 °C for 4 h. A flat piece of PDMS without microfluidic channels was also produced by pouring the polymer on a Petri dish and curing under the same conditions. All PEM coatings were deposited and patterned on the flat PDMS pieces.

PEM Micropatterns. Polyelectrolyte solutions were prepared at a concentration of 1 mg/mL (mol/L (M) concentrations of the repeating units: PAH = 10.7 mM; PEI = 7.7 mM; and PSS = 4.8 mM) with 0.1 M NaCl in 18.2 MΩ water. The pH of the PAH and PSS solutions were 5 and 6, respectively. The PEI solution was adjusted to pH 4.9 with a hydrogen chloride solution (0.1 M). The flat PDMS substrate was first oxidized in an O_2 (approximately 2.6 Pa) plasma for up to 60 s and then brought into contact with the molded PDMS (Figure 1a). The micro-channels were filled with the polycation solution (PEI or PAH) and allowed to stand for 20 min (Figure 1b). The polycation was pumped out of the microchannels using a vacuum, and the channels were rinsed with water and dried. Alternating layers of polyanion (PSS) and polycations were deposited for 5–10 min each, until the desired number of layers was deposited. After the PEMs were patterned, the molded PDMS was removed, exposing a top positive layer on the flat PDMS surface (Figure 1c). The last deposited PEM layer (top layer) was always positively charged (PEI or PAH) to promote cell adhesion.

Atomic Force Microscopy. Atomic force microscopic mea-surements (Dimension 5000, Digital Instruments, Santa Barbara, CA) were acquired in tapping mode to obtain topographical information about the patterned PEM lines. Heights were measured in four different cross-sectional areas of the lines for each number of bilayers. Measurements were made on dried PEMs.

Cell Culture and Patterning on PEMs. Genetically im-mortalized rat retinal R28 cells were established from postnatal day 6 Sprague–Dawley rat retina and contain the 12S portion

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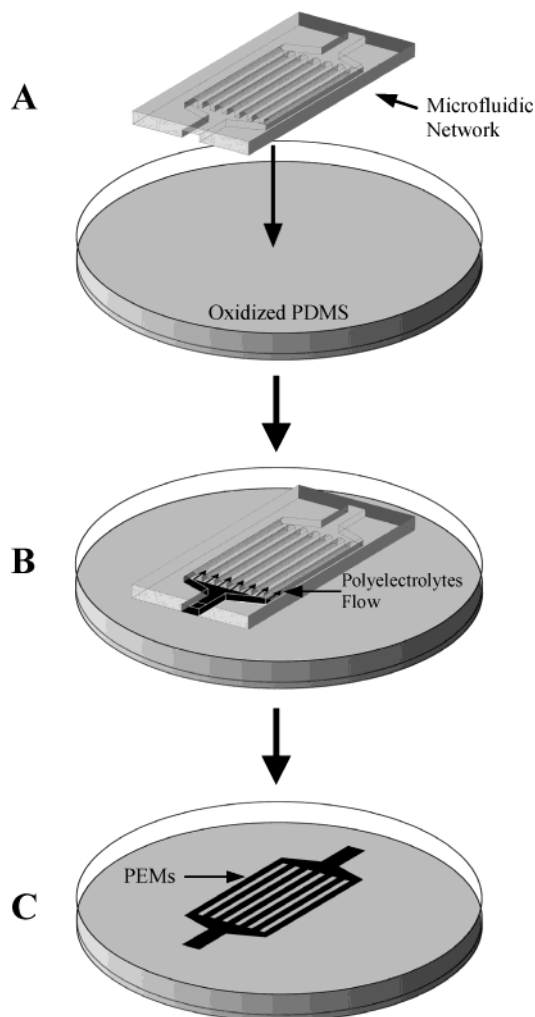
Figure 1. (A) The molded PDMS is brought into contact with a flat, oxidized PDMS surface. (B) Polyions are sequentially introduced through the microchannel network (the black lines show the solution of a polyion filling the channels) and are left for at least 5−10 min inside the microfluidic structure to allow the deposition of the polyion layer. The channel is rinsed with water and filled again with the next polyion. This cycle is repeated until the desired number of layers is obtained. (C) The molded PDMS is removed, and the stacked layers of polyelectrolytes (black lines) are exposed along with the surrounding PDMS surface.

of the adenovirus E1A gene.[24] The 12S portion of the E1A gene was used to promote cell growth, while avoiding tumor-forming potential. R28 cells exhibit contact-inhibited growth, and after intraocular transplantation into rats, no tumor-formation is seen.

Cell Viability Test. After 24 h cell growth on the PEM patterns, a fluorometric assay for cell viability was performed using fluorescein diacetate (DCFDA).[25] The cells were incubated in 10 μM DCFDA (in DPBS) for 30 min at 37 °C in the dark. Fluorescent-viable cells were then examined with an Axiovert 25 microscope (Zeiss, Thornwood, NY), and images were obtained with an AxioCam MRm camera (Zeiss) interfaced with the AxioVision 4.0 software (Zeiss).

Nuclear and Cytoskeletal Staining. Cells growing for 24 h on PEM-patterned substrates were washed with DPBS before fixation in 4% paraformaldehyde solution for 10 min at room temperature. Cells were washed two more times with DPBS. For cytoskeletal staining, cells were incubated in 5 units/mL of phalloidin in 0.1% Triton X-100 (Sigma-Aldrich, St. Louis, MO) for 20 min at room temperature followed by two washes with
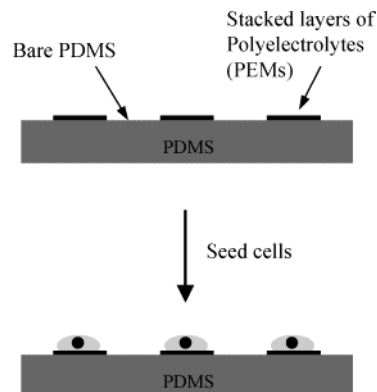
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Figure 2. Side view of PEM lines (positively charged on top) arranged on a PDMS surface. PEMs are spaced in such a way that there are bare PDMS surfaces facing the solution containing the medium and cells. Once cells are seeded, they adhere to the positively charged areas (PEM lines, 50 μm wide).

DPBS. Nuclear staining was carried out after the cytoskeletal staining and consisted of incubating cells in a 5 μg/mL solution of DAPI (in PBS) for 3−5 min at room temperature.[26] After the incubation time, the cells were washed twice with DPBS and covered with fresh DPBS to prevent dehydration. Examination of the cells was achieved using the same equipment and software as described in the previous section.

Results and Discussion

PEMs Deposition and Characterization. The surface properties of cured PDMS have been well-characterized for applications of this material in microfluidic systems and in micropatterning. It was recently reported that the PDMS surface is rendered hydrophilic by exposure to an oxidative plasma but returns to its native hydrophobic state in less than an hour if the oxidized surface is in contact with air rather than water.[27] Therefore, to promote better adsorption of the polyelectrolyte solution to the PDMS via electrostatic interactions, the PDMS was plasma-treated and immediately incubated with the PEM solutions. When the PEM deposition was complete, the PEMs/PDMS substrate was incubated in air, at room temperature, to create a surface with positively charged micrometer-scale lines patterned on a neutral substrate (Figure 2). Micropatterned PEM lines, 50 μm wide and 3.25 cm long, were allowed to dry at least 12 h prior to surface analysis or cell-attachment experiments.

PEM coatings consisting of alternating layers of PEI/PSS or PAH/PSS were produced and characterized by atomic force microscopy (AFM). AFM analysis showed that the thickness (heights) of patterned PEMs correlate with the thickness obtained using a micropatterning convective method that was recently described[28] and with the commonly used immersion deposition method.[29,30] These methods involve the sequential deposition of polyions on silicon and quartz substrates by a shear field (convective method) and the sequential immersion of the substrate in the polyions solutions (immersion deposition method). Thicknesses of up to 19 nm were obtained for 6.5 PEM bilayers (or 13 layers) where one bilayer consists of one polycation layer and one polyanion layer. A linear

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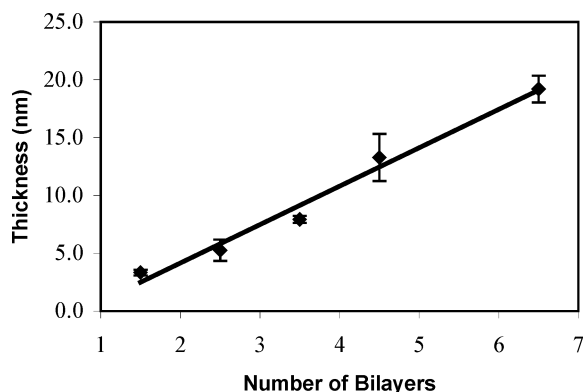(30) Decher, G.; Schmitt, J. *Prog. Colloid Polym. Sci.* **1992**, *89*, 160−164.

Figure 3. Graph of thickness of PEMs as a function of number of bilayers for PEI and PSS multilayers. PEM thicknesses were measured using AFM.

relationship ($r = 0.9905$) between the thickness and the number of bilayers is observed, as shown in Figure 3. These layers were generated at a low NaCl concentration (0.1 M) to obtain smooth and thin PEM lines surfaces.[31,32] It has been reported that, at low salt concentration, similar charges in the polyion chains tend to repel, forcing the chain to extend in a rodlike conformation.[32] If the polyions are then adsorbed onto the substrate surface with a conformation that is similar to the one in solution, the structural shape will render a low surface roughness as well as a thinner film. It has already been observed that high surface roughness and thicker PEM films are obtained with high salt concentrations.[32] In addition, the pH of the polyelectrolyte solutions was adjusted so that the percent of polymer chain charges was 50% or more.[33] Fully charged polymers tend to arrange in a less random coil conformation and, therefore, form more compacted PEM structures.[20]

Cell Micropatterning. Cells have a negatively charged surface as a result of the glycoproteins and glycolipids located on the external surface of the plasma membrane.[34] PEI and PAH are polymers containing amine groups that are partially charged at the pH used in these experiments. Rat retinal R28 cells were seeded on the PEMs/PDMS surface at a concentration of ~250 000 cells/mL (25 500 cells/cm²). R28 cells typically adhere to the polystyrene culture-flask surface between 4 and 8 h after seeding. Cell adhesion on the PEMs was comparable to tissue-culture plastic and observed by 5.5 h after seeding. As shown in Figure 4A, by 24 h in culture, R28 cells on a polystyrene culture-flask surface were well attached, and neurite-like extensions were already projecting from the cell body. When seeded on PEI and PAH, the cells have attached and spread after 5 h (Figure 4B and C). Neurite-like processes were observed to grow within 24 h on both polycations (white arrows, Figure 4C and D). As shown in parts B–E of Figure 4, the cells tended to align along the PEM lines. Rounded (unattached) cells are observed on bare PDMS areas between the positively charged patterned lines. In these experiments, the PDMS surface that delimited the PEM patterns was left bare; that is, no blocking agent or physical barrier was used to prevent
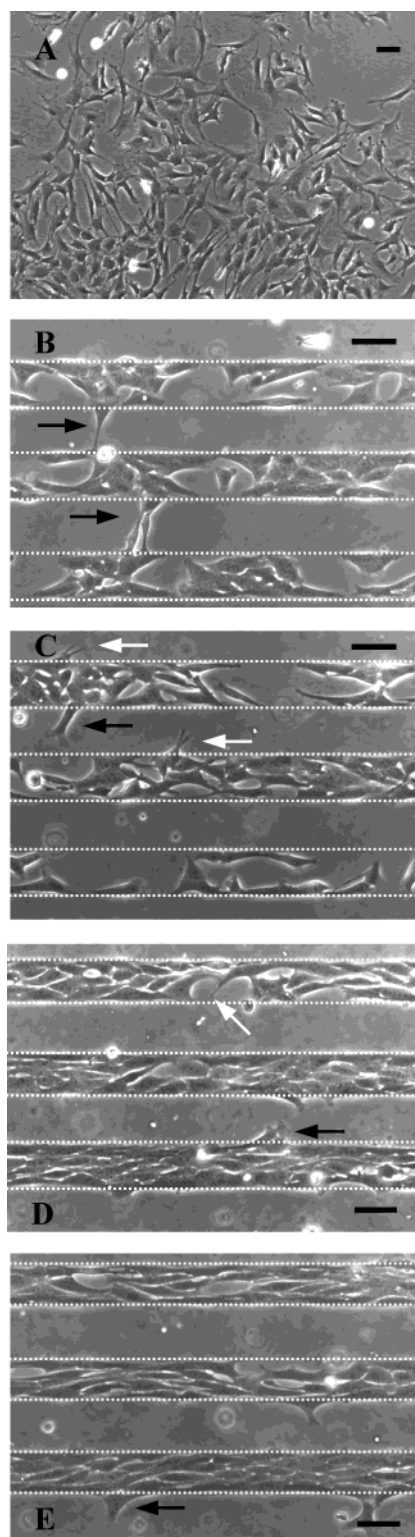

Figure 4. (A) R28 cells growing on polystyrene culture flask (24 h after seeding). (B) and (C) are R28 cells 5.5 h after being seeded on PAH and PEI (11 layers), respectively. Dashed white lines represent the PEM regions and are about 50 μm in width. (D) and (E) are R28 cells 24 h after seeding on PAH and PEI (11 layers), respectively. All scale bars are 50 μm.

cell adhesion onto the PDMS surface. It is believed that polar groups on the surface of PDMS travel toward the bulk when the surface is exposed to air[35] and should revert back (to the surface) when the PDMS is returned to the

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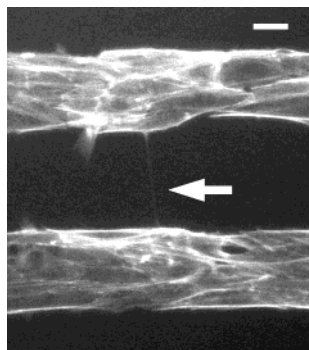
Figure 5. Fluorescence image of a small membrane tube (arrow) connecting two cells in adjacent PEM lines (PAH on top, 11 layers). Cells were stained with phalloidin, and the fluorescent image shows the presence of actin in the cytoskeleton and in the small tube. The tube is a few hundred nanometers in diameter. Scale bar: 20 μm.

Figure 6. Two weeks growth of R28 cells on 50 μm-wide PEI lines (13 layers). Insert picture is a close view showing excessive growth leading to the formation of aggregates on the PEMs and extensions between the lines. Scale bar: 4.2 mm.

aqueous environment of the cell media. If in fact this is occurring, negatively charged surface areas should be emerging in the nonpatterned regions of the PDMS upon the addition of the cells. However, cell adhesion in the unpatterned regions was initially minimal and was primarily confined to the positively charged PEM regions. This behavior is expected due to the dramatic improvement in the adhesive properties shown by culture surfaces (in reduced serum conditions) when a charged surface is altered from negative to positive.[36−38] As the availability of free PEM surface progressively diminished, a minority of R28 cells began to spread onto the uncharged PDMS regions (black arrows, Figure 4D and E). The survival of these cells on uncharged PDMS appeared to be dependent on the continued contact of the cells with one edge or opposing edges of the PEM patterns (black arrows, Figure 4B and C). This is supported by the observation that cells on PDMS, which had no contact with either edge of the PEM pattern, remained rounded-up and did not fluoresce during the viability assay.

Nanotubes connecting cells in culture, referred as tunneling nanotubes (TNTs), were recently discovered.[39] These TNTs behaved as intercellular membrane channels used to transfer membrane vesicles and organelles. TNTs were found to be formed de novo, to contain actin, and to be continuous with the cell membrane of connected cells. Cells on PEM lines connecting to cells on adjacent PEM lines, via small tubes that resemble the TNTs recently reported, were also observed in our experiments. These tubes had dimensions of a few hundred nanometers in diameter and up to 55 μm long. Staining of these small tubes revealed that they contain actin (arrow, Figure 5). It is not known what triggers the generation of TNTs toward neighboring cells. In the case of cytonemes, another type of cellular extension, it is believed that long-range concentration gradients of a chemical signal are responsible for the growth of such structures.[40] It is likely possible that a chemical signal might be inducing the growth of TNTs, but to the best of our knowledge, this has not been studied yet. The finding of these tubes in patterned cells suggests that the growth of the small tubes could be studied in an ordered fashion by controlling the distance between cells and by finely controlling the chemical
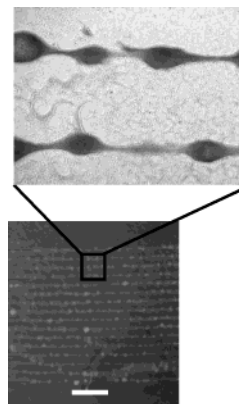
environment surrounding the cells, if patterned within microchannels.

R28 cells were cultured for up to two weeks on PEI surfaces (Figure 6). In this case, cell bodies aligned and grew along the PEI patterned lines. Although there were no cell bodies on the uncoated PDMS substrate, cells on PEI grew neurite-like extensions that projected along the PEMs but also between the PEMs onto the PDMS. Processes that projected from the PEM lines to the PDMS substrate were able to connect to cells on adjacent patterned lines.

Cell viability of R28 cells was tested 24 h after seeding and is shown in Figure 7 for cells grown on standard tissue-culture polystyrene (Figure 7A) and on the PEMs (Figure 7B and D). Panels C and E of Figure 7 show a micrograph of the same area where the viability test on R28 cells was performed. Basically, all cells adhered on the PEMs were viable, and the dark areas in Figure 7B and D are areas where cells were absent. The intracellular oxidative activity of the cells was measured by loading DCFDA, dissolved in DPBS, into the cells. If cells are viable, oxidation of DCFDA is carried out by intracellular esterases cleaving DCFDA to produce carboxydichloro-fluorescein. This fluorescent product is observed intracellularly when the cells are alive. Figure 7A shows the fluorescent products of the intracellular oxidation of DCFDA by viable retinal R28 cells on polystyrene. Cell viability on a number of PEM layers, ranging from 1 to 11 layers, was assayed. Panels B and D of Figure 7 show viable R28 cells on PAH and PEI micropatterns (11 layers), respectively, 24 h after seeding. R28 cells on 1, 3, and 7 micropatterned layers were also shown to be viable (data not shown).

The actin filaments are the major structural proteins underlying cellular architecture. We labeled the cytoskeleton of R28s to indicate whether cellular morphology is preserved in cells cultured on PEMs. Figure 8A shows a fluorescent image of rat retinal R28 cells adhered on polystyrene. Actin filaments are stained red, and the nuclei are stained blue. The morphology of the cells on polystyrene seemed to be similar to the morphology on PAH (Figure 8B) and PEI (Figure 8C), particularly on the areas where the cells had space to spread more freely. In confined regions on the micropatterned substrate, the cells tended to stretch and become elongated in order to accommodate more cells within the cell adhesive pattern lines. Mostly rounded nuclei were observed on polystyrene (Figure 8A) and on areas of PAH and PEI that had dimensions greater

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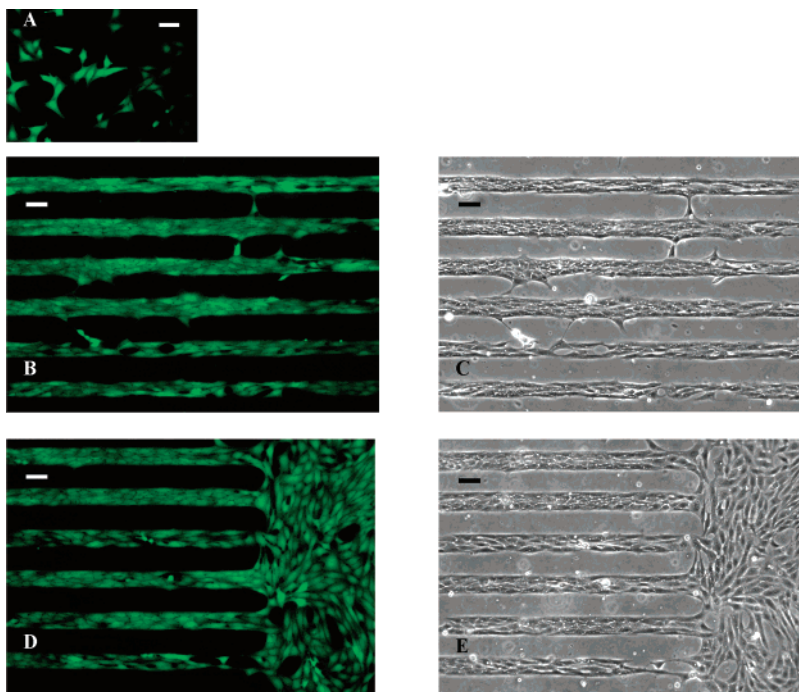(40) Ramirez-Weber, F. A.; Kornberg, T. B. *Cell* **1999**, *97*, 599−607.

Figure 7. Viability assay showing the fluorescent product of the intracellular oxidation. Viable retinal R28 cells adhered on polystyrene (A) are observed. R28 cells patterned on PAH (B) and PEI (D) show cellular viability as observed by the green fluorescence. (C) and (E) show the micrographs that correspond to the fluorescence images (B) and (D), respectively. All patterned cells are viable. Scale bars: 50 μm.
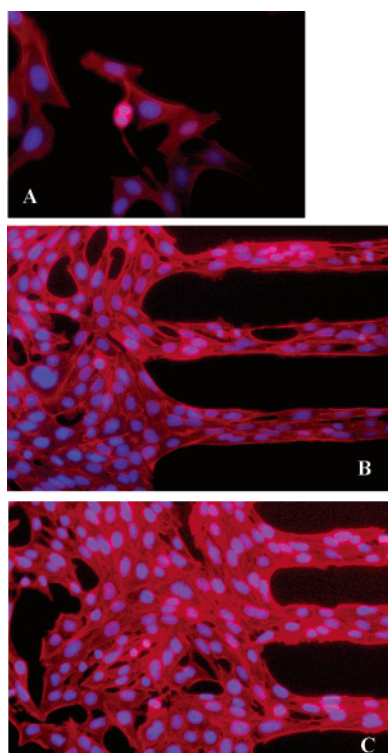

Figure 8. Fluorescent images of the cytoskeleton and nuclei of R28 cells on polystyrene (A), PAH (B), and PEI (C). The cytoskeleton is denoted by the red color, whereas the nuclei are observed in blue color. The morphology on polystyrene and the larger areas of PAH and PEI are similar. Cells are elongated when patterned in smaller areas as observed on the PEM lines ((B) and (C)).

than 50 μm. On the other hand, elongated cells on the lines tend to have a more elongated nuclei shape.

Conclusions

This work shows a simple method of patterning poly-electrolyte multilayers on PDMS surfaces and the selective adhesion of neuronal (retinal) cells on the patterned PEM features. By using microfluidic networks, selective areas on the PDMS were patterned with polyelectrolyte layers. By allowing the bare PDMS surfaces to return to the neutral state, the need for blocking agents to prevent cell adhesion in areas outside the PEM lines was unnecessary. Retinal cells tended to attach on the positively charged PEM lines (PAH and PEI) and not on the PDMS areas, which serve as cell-resistant surfaces. They were allowed to proliferate for up to two weeks without a visible decrease in adhesion capacity. Viability of the cells, following adhesion on PEI and PAH, was assessed as high on both surfaces. This result proved that the cells were alive and that the polymers were not toxic to these types of cells. This finding is important since it was recently reported that PEI is toxic to bone cells.[16] Also, cell viability was tested on different numbers of PEM layers, producing the same results. The morphology of the cells on the PEMs was examined as well. No apparent differences were observed on the cytoskeleton of the retinal cells when compared to the adhesion on polystyrene and on the PEI and PAH surfaces with dimensions greater than 50 μm, whereas cell elongation was observed on PEM surfaces with dimensions of 50 μm. The use of PDMS as the patterning-tunable surface, along with the use of non-biological polymers for cell patterning, should allow the fabrication of devices that will last longer once the pattern has been created. Furthermore, preventing the use of blocking agents, along with the use of biocompatible material, promises a rather off-the-shelf micropatterned system.