Facilitating the Culture of Mammalian Nerve Cells with Polyelectrolyte Multilayers

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Received November 30, 2005. In Final Form: March 2, 2006

When neuron-like cells (NLCs) derived from pluripotent embryonal carcinoma cells (P19) are cultured on bare tissue culture substrates, they require a monolayer of fibroblast cells to exhibit normal neurite outgrowth, behavior typical of neuronal cultures. However, substrate treatment with polyelectrolyte multilayers (PEMs) composed of poly(allylamine hydrochloride) (PAH) and poly(styrenesulfonic acid) (PSS) significantly improved these cultures. Cell morphology was more spread, indicative of healthy cells, and direct attachment of neuronal cell bodies to the treated surface was observed. Neuronal outgrowth across the surface was not dependent on an underlying fibroblast monolayer with the PEMs surface treatment. Additionally, the PEMs surface treatment can be used to condition various surfaces, facilitating neuronal cultures on surfaces which are natively hydrophilic (tissue culture polystyrene) or hydrophobic (poly(dimethylsiloxane), PDMS). Microfluidic networks were used to micropattern the PEMs onto PDMS, resulting in confined regions of cellular attachment and directed neuronal outgrowth. The ability of PEMs to encourage NLC attachment without supporting cells to a variety of surfaces and surface geometries greatly simplifies neuronal culture methodology and enables neuronal investigations in new environments.

Introduction

Cell culture is a powerful *in vitro* tool for studying cell behavior, investigating cellular responses to various stimuli, determining drug efficacy and toxicity *ex vivo*, and facilitating drug discovery. Immortalized cell lines are available for most types of mammalian cells, as well as for many specific disease states. These provide genetic homogeneity that simplifies cell culture experiments by minimizing uncontrolled experimental variables. However, the bulk of research on neuronal cells still utilizes cultures or primary cells, where central nervous system (CNS) tissue is extracted from a sacrificed animal. These cultures are expensive, requiring the maintenance of live animal stocks, and are prone to significant variability between animals despite efforts to control all environmental factors.[1,2]

Despite the significant health care impact of neurodegenerative diseases such as Alzheimer's, Huntington's, and Parkinson's, there are few useful cell lines with which to study these diseases. Developing neuronal cell cultures proves inherently difficult because neuronal cells are characteristically postmitotic.[3] Additionally, neurons *in vivo* are exceedingly specialized and typically depend on nonneuronal glial cells for maintenance of extracellular homeostasis.[4] The immortalized neural cell lines that do exist are generally derived from neural tumors.[3] Although these cells exhibit mitosis and can be immortalized, they are inherently abnormal neuronal cells, since they exhibit cellular division. Even these cell lines will often only survive and develop neurites when cultured on a monolayer of glial cells.[2]

An alternate approach is to maintain a stock of neuronal precursor cells that continue to divide prior to differentiation, but can be differentiated to produce stable neural cell cultures. One such cell line is the embryonal carcinoma (P19) cell line. P19 cells are rapidly dividing, but can be induced to differentiate terminally along CNS, skeletal muscle, or cardiac muscle pathways.[5] When treated with retinoic acid, a compound important for the development of the embryonic brain, P19 cells differentiate stably into many different CNS cell types including GABAergic and glutaminergic neurons, fibroblast cells, and glia.[6−8] The NLCs derived through P19 differentiation are characteristically postmitotic, exhibit neurites that project across a layer of genetically identical fibroblast cells, generate spontaneous and stimulated action potentials, and form synapses.[6]

Polyelectrolyte multilayers (PEMs) have been used successfully to control cellular attachment to various surfaces.[9−19] Virtually

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10.1021/la053244b
Published on Web 05/19/2006

any charged surfaces can be modified with PEMs to mask and stably alter the substrate properties.[20] Choosing PEMs amenable to cell attachment enables cell culture on otherwise adverse substrates, and these PEMs have been used previously to pattern the surface attachment of immortalized retinal cells.[11] Here we utilize PEMs to facilitate NLC cultures by enabling direct attachment of NLC cell bodies to the surface and neuronal projections across the PEMs treated surface. Various surfaces can be successfully treated with the PEMs with improvements to cell culture. Additionally, micropatterned PEMs surfaces can be used to constrain the geometry of neurite outgrowth.

Experimental Section[15]

Materials. Cell culture media (Minimum Essential Medium), serum (fetal bovine and newborn calf), and trypsin-EDTA (1X) were purchased from Invitrogen Corporation (Carlsbad, CA). Both antibodies (mouse anti-rat-neurofilament (160 kD) and FITC (fluorescein isothiocyanate) labeled rabbit anti-mouse-IgG) and trans-retinoic acid (≥ 98% purity) were from Sigma-Aldrich (St. Louis, MO). P19 cell stock (CRL-1825) was obtained from American Type Culture Collection (Manassas, VA). Bacteriological grade and tissue culture (#430293) Petri dishes were from Fisher (Fairlawn, NJ) and Corning (Acton, MS), respectively. Poly(allylamine hydrochloride) (PAH, $M_W \approx 70\,000$) was purchased from Sigma-Aldrich (St. Louis, MO), and poly(styrenesulfonic acid) (PSS, $M_W \approx 70\,000$) was purchased from Polysciences, Inc. (Warrington, PA). Poly(dimethylsiloxane) (PDMS, Sylgard 184) was from Robert McKeown Co., Inc. (Branchburg, NJ).

Cell Culture and Differentiation. The P19 cell line was maintained using standard cell culture techniques and published methods.[5] Briefly, cells were cultured in media with added serum. Prior to confluency (every 2−3 days), the cells were suspended using trypsin−EDTA and passed (1:10 dilution). The culture was sustained through no more than 15 passes overall; a cell stock after the first pass was kept frozen under liquid nitrogen. Thawed cells were passed at least twice prior to experimentation to ensure an appropriate number of viable cells. P19 cells were induced to differentiate along a CNS pathway by exposure to retinoic acid (10^{-6} mol/L) at high cell densities for 4 days. High cell densities were achieved by placing the suspended cells in bacteriological grade Petri dishes where they aggregated spontaneously. The induced aggregates were plated directly (see Figure 1) or broken up into dispersed cell suspensions prior to plating by treatment with trypsin-EDTA and agitation. In both cases, cultures were plated at 3.75×10^5 cells/mL and were comprised of many CNS cell types including NLCs, glia, and fibroblasts.

PEMs Surface Treatment. Polyelectrolyte multilayers (PEMs) were generated through layer-by-layer deposition.[21] Briefly, polyanions (PSS) and polycations (PAH) were sequentially adsorbed onto a charged surface.[11] To ensure a smooth surface, complete surface coverage, and a strong electrostatic interaction between the surface and first polyelectrolyte layer, solutions were prepared at low salt concentration (0.1 mol/L NaCl), filtered (0.2 μm), and used immediately. For tissue culture-treated substrates, PSS was deposited first (1 layer), and a total of 6 alternating polyelectrolyte layers were used. PDMS substrates were first oxidized in an oxygen plasma to render the surface negatively charged. Then, the PAH was deposited first, and a total of 5 layers were used. In both cases, the top polyelectrolyte layer was the polycation. The PDMS surface charge following plasma oxidation is unstable and degrades rapidly (hours) to the native PDMS surface.[22] Since the surface coverage of the PEMs depends on the substrate surface charge, PEMs were deposited immediately following PDMS oxidation. As reported previously,[11] the PEMs surface treatment exhibited significant stability on PDMS, persisting far longer than the charge on oxidized but untreated PDMS. The prepared surfaces could be dried, stored for weeks, and exposed to sterilizing UV irradiation prior to experiments.

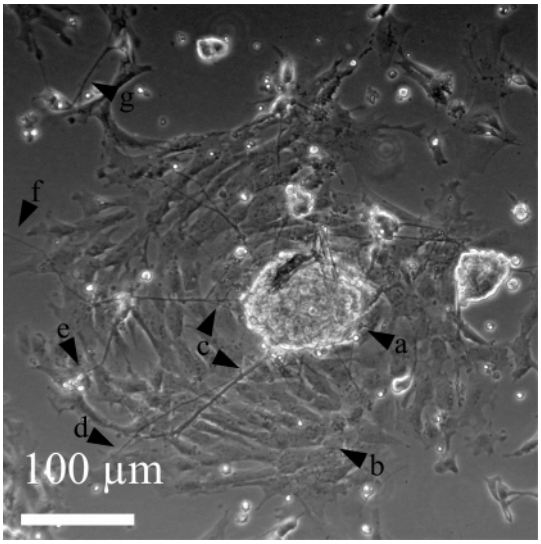

Figure 1. NLCs plated as aggregates. One day after plating NLC aggregates (a), fibroblast cells (b) spread outward across the tissue-culture polystyrene surface forming a monolayer which neurites (c) project across.[6] Projections terminate at the edge of the fibroblast monolayer (d and e) or project linearly onto nearby adherent cells (f and g).

Surface Patterning. PEMs deposition was spatially confined using temporary microfluidic channels as described previously.[11] Masters containing the desired geometry of channels were patterned photolithographically using SU-8 photoresist (15 μm to 40 μm thick) on glass. PDMS was cured against this master and removed to yield the pattern in negative relief. Under slight pressure, the patterned PDMS adhered conformally to a planar PDMS slab prepared separately, forming microfluidic channels. Polyelectrolytes were hydrodynamically pumped through these channels with intermittent water rinses to generate PEMs. Removal of the microfluidic channels left the PDMS slab with micropatterned regions of PEMs surface treatment.

Microscopy. An Axiovert 25 (Zeiss, Thornwood, New York) inverted microscope was used to image cells in brightfield using phase contrast microscopy. Also, the edge of the micropatterned PEMs surface was faintly visible. The PDMS surfaces had a high index of refraction and were sometimes fairly thick (≈5 mm), leading to diminished contrast with phase contrast optics. The NLCs were fluorescently labeled using a double immunoassay for neurofilaments.[6] Fixed cells were first incubated with mouse anti-rat neurofilaments (primary antibody). Subsequently they were incubated with FITC-labeled rabbit anti-mouse IgG (secondary antibody). Fluorescence microscopy was used to image the labeled cells.

Results

Neuronal Projections on Tissue Culture Substrates. The induction process which produces differentiated NCLs also produces glia and fibroblast cells in large cell aggregates which are 100−300 μm in diameter and contain several hundred

(15) Certain commercial equipment or materials are identified in this report to specify adequately the experimental procedure. Such identification does not imply recommendation or endorsement by the National Institute of Standards and Technology, nor does it imply that the materials or equipment identified are necessarily the best available for the purpose.

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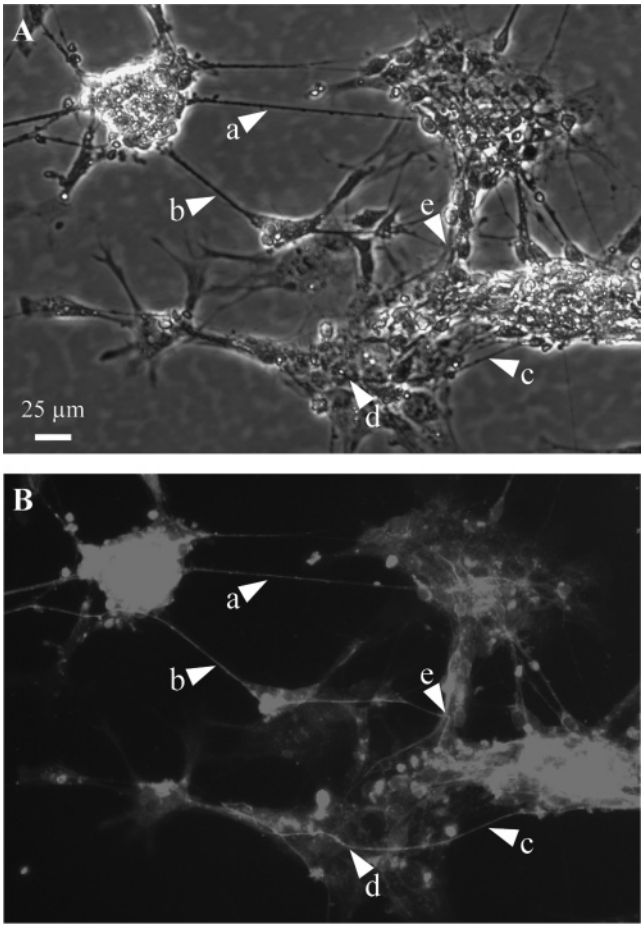

Figure 2. Monodispersed NLC cultures. Four days after plating monodispersed NLCs, neuronal outgrowth was imaged with bright field (A) and fluorescence (B) following a double immunoassay for neurofilaments. Projections across the tissue culture substrate are linear (a−c) while projections across other cells are convoluted (d and e).

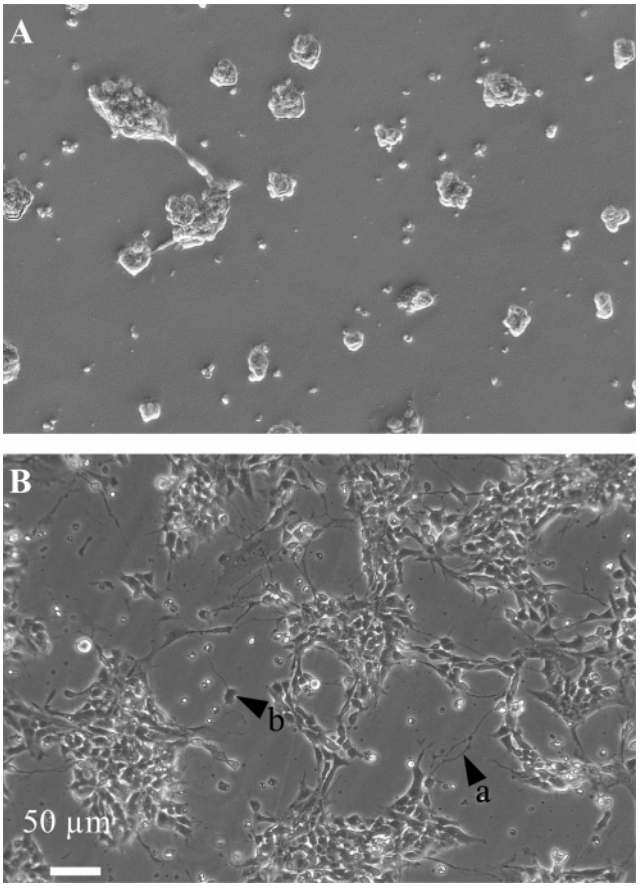

Figure 3. PEMs treated tissue culture. Monodispersed NLC cultures 1 day after plating on bare tissue culture (A) and PEMs treated tissue culture (B) substrates. Neuronal projections (a) and NLC cell bodies (b) were observed on the PEMs treated surface without underlying adherent cells.

individual cells. When plated on tissue culture-treated substrates, these aggregates sedimented and attached to the surface (Figure 1a) over the course of 4−8 h. Fibroblast cells then spread outward across the substrate forming a tight monolayer of cells surrounding the aggregate (Figure 1b). After 3 days in culture, this fibroblast monolayer was continuous across the surface. Although neuronal cell bodies remained inside the aggregate, significant neurite outgrowth onto the fibroblast monolayer (Figure 1c) was observed after only 1 day. Although these projections could extend several hundred micrometers, their growth was confined by the geometry of the fibroblast monolayer (Figure 1d,e). Neuronal projections were not found terminating on the bare tissue culture substrate. Where there were discontinuities in the fibroblast monolayer, neuronal projections were observed extending across the bare tissue culture surface. However, these projections always connected to nearby fibroblast cells, not directly to the tissue culture surface (Figure 1f,g).

Alternately, cell aggregates were broken up and plated as a dispersed cell suspension. Subsequently, some aggregation occurred as cells attached to the tissue culture surface, yielding small cell clusters (tens of cells, Figure 2A). Although the overall cell density was identical to the cultures of whole aggregates, fibroblast cells took longer (5 days) to spread and achieve surface coverage in dispersed cultures, and neuronal outgrowths were not observed until after 3 days in culture. As in aggregate cultures, neuronal projections never terminated on the bare tissue culture surface, though they could extend to nearby surface-attached cells. Additionally, where the neurites extended across the bare surface, their geometry was invariably linear (Figures 1f,g and 2A,a−c).

Identification and tracking of neuronal outgrowths over other cells was occasionally difficult using phase contrast microscopy and cell morphology. Immunofluorescent labeling of neurofilaments in fixed cell cultures facilitated visualization of neuronal outgrowths and allowed discrimination of NLCs from other cell types (Figure 2B). Although neuronal projections across the tissue culture surface were invariably linear (Figure 2a−c), their path was generally quite tortuous when attached to other adherent cells (Figure 2d,e). Linear projections could also be observed extending over other cells, mostly in regions of significant surface topology. For example, when neurons project from the center of a cell aggregate (≈100 μm tall) to a thin cell monolayer, they would take the shortest path between two points of attachment by extending linearly out of the plane of the surface (Figure 1c).

Surface Treatment with Polyelectrolyte Multilayers (PEMs). When tissue culture Petri dishes were treated with PEMs to improve cellular attachment and growth, the treated substrates exhibited lower static contact angles for aqueous solutions than untreated surfaces (Table SI1 in the Supporting Information). Induced, monodispersed P19 cells were plated onto the PEMs surfaces and compared to bare tissue culture dishes (Figure 3). After 1 day of culture, cells on the untreated surface exhibited some aggregation, as noted previously, and many individual cells remained entirely detached from the surface. Adherent cells exhibited poor spreading across the surface, as indicated by their

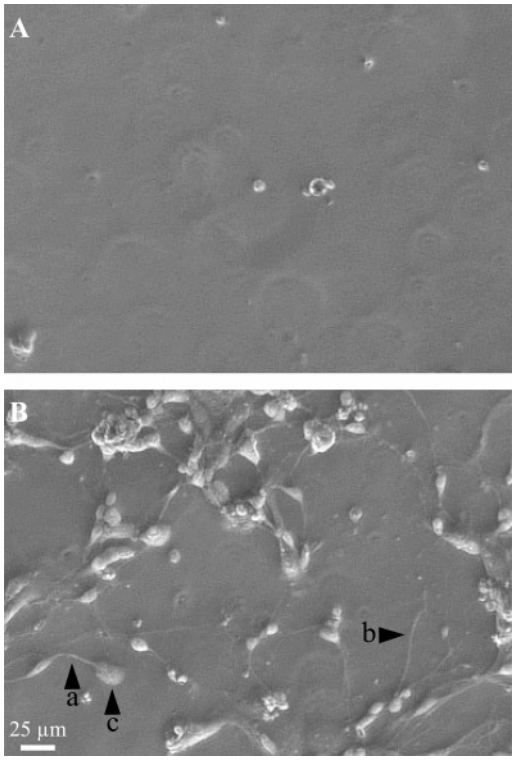

Figure 4. PEMs treated PDMS. Monodispersed NLC cultures 3 days after plating on native PDMS (A) and PEMs treated PDMS (B). Neuronal projections (a and b) and NLC cell bodies (c) were observed on the PEMs treated surface.

bright halo in phase contrast imaging, and no neuronal projections were observed (Figure 3A).

In contrast, cells on the PEMs treated tissue culture surface exhibited significantly less aggregation, with fewer nonadherent cells, and individual cells were well spread, appearing phase-dark (Figure 3B). Extended neuronal outgrowth was seen after just 1 day of culture, similar to cultures of whole aggregates where the fibroblast monolayer rapidly provided a suitable surface for neurites. Notably, neuronal projections were observed in the absence of underlying fibroblast cells (Figure 3B,a). Unlike neuronal outgrowths over bare tissue culture surfaces, these projections were not invariably linear. Further, NLC cell bodies could attach directly to the PEMs treated surface (Figure 3B,b). As a control, the PEMs surface treatment was applied to one part of a culture dish while the rest remained untreated. The culture away from this modified region was indistinguishable from cultures on wholly untreated substrates, confirming that the PEMs treatment remained adsorbed to the surface and spatially localized.

PEMs were also deposited onto PDMS to yield a cell culture surface. Native PDMS is inherently hydrophobic and becomes coated with albumin when exposed to the serum-containing media used in cell culture.[23] Even after several days, induced and dispersed P19 cells showed very little cellular attachment or growth on bare PDMS surfaces (Figure 4A). In contrast, both attachment and growth were seen on PEMs treated PDMS, albeit slower than on similarly treated tissue culture surfaces (Figure 4B). The cells exhibited minimal aggregation as they attached and had a well spread morphology on the PEMs treated surface. Projections from NLCs were not confined by the spreading fibroblast layer, but could extend across the treated PDMS surface (Figure 4B,a,b). As in the case of PEMs treated tissue culture

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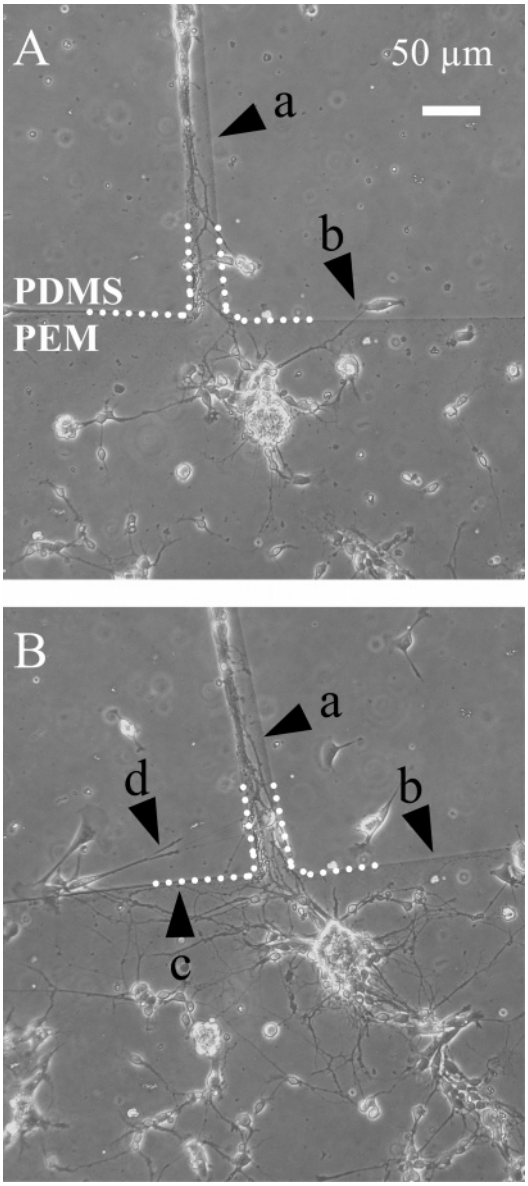

Figure 5. Micropatterned PEMs on PDMS. Monodispersed NLC cultures two (A) and three (B) days after plating on a micropatterned surface. The bottom of each image as well as the 20 μm wide vertical line are PEMs treated surfaces. NLC projections are confined along the narrow PEMs line (a) and by the edge of the PEMs surface treatment (b and c). Projections across the untreated PDMS surface were linear (d). Dotted white lines are added to clarify the intersection of the two PEMs treated areas.

surfaces, NLC cell bodies attached directly to the PEMs treated PDMS (Figure 4B, c), and projections across the surface could be quite tortuous. Since the untreated PDMS exhibited minimal cell attachment or growth, the contrast between cell cultures on untreated PDMS and PEMs treated PDMS was significant.

Micropatterned PEMs Treatment. PEMs treatment of the oxidized PDMS surface was spatially patterned using removable microfluidic networks. As reported previously, the untreated PDMS returned to its native hydrophobic state after several hours exposed to air.[11,22] This produced a generally hydrophobic surface with micropatterned PEMs treated areas amenable to cell growth (Figure 5). When induced, monodispersed P19 cells were plated onto the micropatterned surface, cells exhibited minimal immobilization on the untreated PDMS after 2 days, whereas in the PEMs treated regions, surface attachment as well as NLC projections were readily observed. Cell growth on PEMs away

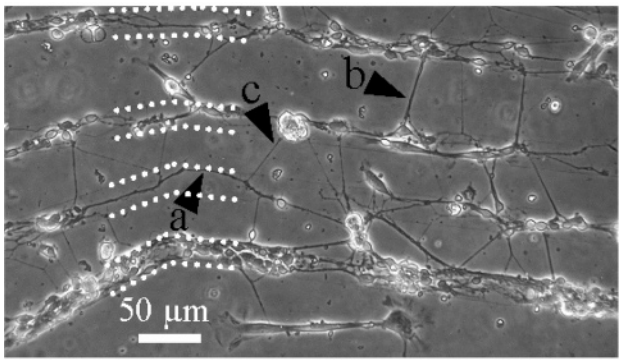

Figure 6. Discontinuous PEMs micropattern. PEMs surface treatment was confined to four nonintersecting lines 20 μm wide. Three days after plating, neuronal projections bend (a) or project linearly across the bare PDMS, attaching either to other cells (b) or to the PEMs treated surface (c). Dotted white lines are added to clarify the edge of the PEMs lines at the bend.

from the untreated PDMS was indistinguishable from bulk cultures on PEMs treated PDMS. However, near the interface between PEMs treated and untreated areas, NLC projections across the surface were confined by the geometry of the PEMs surface treatment (Figure 5A,a,b). After 3 days of culture, the coverage of NLC projections across the PEMs surface were significant, with minimal extension beyond the geometry of the micropatterned surface treatment (Figure 5B,b,c). Even when many projections were tightly confined on a narrow line, there was no deviation onto the native PDMS surface (Figure 5B,a). Where the projections did extend across the bare PDMS, such as near inside corners in the micropatterned geometry, they were invariably linear and terminated on other cells (Figure 5B,d). This was qualitatively similar to neuronal projections across the untreated tissue culture surface between fibroblast cells.

When PEMs were deposited in narrow unconnected regions (20 μm wide) across the PDMS surface, cellular attachment was crowded onto the treated areas (Figure 6). NLC projections along the PEMs treated regions followed bends in the pattern, avoiding the untreated PDMS (Figure 6,a). Alternately, NLC outgrowths could extend onto neighboring PEMs regions across the untreated PDMS; however, such projections were invariably straight. The NLC projections across the bare PDMS attached to either other cells (Figure 6,b) or directly onto the PEMs surface (Figure 6,c).

Discussion

When P19 cells are induced to differentiate into NLCs, they produce a complex culture of many cell types including post-mitotic NLCs, glia, and fibroblasts.[5,6] When compared to immortalized neuronal cell lines with limited cell types and dividing NLCs, this system provides a better model for *in vivo* complexity. When compared to primary cultures from animals, differentiated P19 cell cultures offer less variability and decreased cost. However, on tissue cultures plates, the P19-derived NLCs exhibited neuronal outgrowth that was confined by the geometry of the fibroblast cell monolayer. When fibroblast spreading was slowed, as with dispersed cultures, projections from induced neuronal cells were not observed for 3 days. The aggregation observed when plating dispersed suspensions of induced cells indicates that cells are more likely to attach to already-adherent cells than directly to the tissue culture surface. This behavior is not uncommon for neural cells, and culture protocols for neural cells often prescribe growth over an underlying cell monolayer which provides a suitable surface for neuronal attachment and neurite outgrowth.[2] While allowing neuronal cultures, this cellular monolayer complicates culture procedures and can impair microscopy of the processes (particularly for transmitted light). Staining for neurofilaments (Figure 2) facilitates imaging of neuronal processes across other cells but requires fixation of the cells and is incompatible with live cell imaging. It would be very useful to have a facile surface treatment for generating surfaces amenable to direct neuronal attachment and outgrowth.

PEMs are rapidly ($<$1 h) deposited in a layer-by-layer (LBL) process yielding novel surface properties.[20] Cells plated onto the PEMs treated tissue culture surface attached with minimal aggregation, indicating favorable surface interaction. Additionally, the adherent cells were well spread, a morphology generally indicating healthy cells with good surface attachment. Significantly for neuronal cultures, NLC cell bodies also attached directly to the PEMs treated surface (Figure 3B,b). For tissue culture substrates, PEMs treatment allowed neurite outgrowth for dispersed neuronal cultures in a time comparable to cultures of whole aggregates ($<$24 h), much sooner than for dispersed cultures on untreated substrates (2−3 days). With PDMS substrates, PEMs surface treatment enabled NLC cultures where no growth was otherwise detected. Additionally, projections across PEMs treated surfaces could be quite tortuous and highly branched (Figures 3B,a and 4B,a), suggesting that these projections readily formed multiple sites of attachment directly to the substrate. In contrast, neuronal projections on untreated tissue culture surfaces were notably linear (Figure 2,a) and only exhibited multiple points of attachment when passing over adherent cells, indicating little or no direct attachment to the surface. Indeed, PDMS substrates are expected to resist cellular attachment, and when the PEMs treatment was micropatterned onto PDMS surfaces, neuronal projections extending across the untreated PDMS were similarly linear (Figure 5B,b).

The improvement in NLC culture for the PEMs treated surfaces can be attributed in part to improved surface attachment. The PEMs surface treatment yields a highly charged substrate, as evidenced by contact angle measurements demonstrating increased hydrophilicity as compared to bare tissue culture or PDMS substrates (see Table SI1 in the Supporting Information). Electrostatic attraction between the polycation and the net negative charge on the cell surface probably provides the initial surface interaction, but persistent attachment and growth involves more complicated biochemical interactions. Free protonated amines in the final PAH layer may be important as primary amines have been reported to encourage neuronal growth when covalently bound to a surface.[24] Consistent with this, PEMs surface treatment using another polycation containing only secondary amines (polyethyleneimine, data not shown) provided mixed results for P19 NLCs, with some improved surface attachment but minimal surface growth. Alternately, investigations of attachment of other cell types to PEMs have indicated a variety of important factors including mechanical compliance, solvent swelling, hydrophobicity, and topology of the surface in addition to surface chemistry.[10,12−19] As the PEMs surface treatment utilized here changes both the physical and chemical characteristics of the surface, more investigations are necessary to determine the most important elements for encouraging attachment and growth of neurons to PEMs surfaces. The particular PEMs composition utilized here was chosen based on its initial success for attachment and growth of a cell line of immortalized neural cells[11] and continued investigations may yield further improvements for NLC cultures.

The differences in the current investigation between PEMs deposited on tissue culture and oxidized PDMS substrates may

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be attributed to the low number of polyelectrolyte layers used (5 or 6 layers). Using atomic force microscopy (AFM), the micropatterned PEMs thickness on PDMS was determined to be 5.8 ± 0.2 nm with a surface roughness increase from 5.0 to 8.3 nm rms (see Figure SI1 in the Supporting Information). Considering the large surface roughnesses for these devices, it is difficult to determine the homogeneity of the PEMs surface treatment. A larger number of polyelectrolyte layers could be used to better screen any effect of the untreated substrate. PDMS is a particularly useful material that is hydrophobic in its native state, providing a marked contrast between PEMs treated and untreated regions and is easy to engineer, facilitating fabrication of microfluidic systems via soft lithography.[21] The PEMs surface treatment demonstrated here could facilitate the further development of biomimetic microenvironments for neuronal investigations.[25−27]

The PEMs surface treatment was readily patterned using removable microfluidic networks, and NLC attachment was limited to the PEMs treated regions of PDMS substrates (Figures 5 and 6). The PEMs micropattern also limited neuronal outgrowth, causing the processes to bend in order to remain on PEMs patterned regions (Figure 6,a). Neurites could extend across the untreated PDMS, though attachment to this surface seemed poor. To our knowledge, this is the first use of PEMs to confine or guide the growth of neuronal projections. Unlike alternate surface treatments which depend on biological molecules,[28−30] the PEMs treated surfaces were extremely robust and could be left exposed to air for several weeks and/or sterilized by exposure to UV light prior to these experiments with no deleterious effect.

Conclusions

PEMs surface treatment facilitated cell cultures of induced P19 cells. Many different substrates can be treated with PEMs, allowing NLC cultures even on substrates otherwise adverse to cellular attachment and growth (e.g., PDMS) and improving cultures on commercial tissue culture substrates. The deposition methodology allowed facile patterning of the surface, simplifying the neural networks formed in culture and giving some control over the geometry of neurite outgrowth. PEMs surface treatment facilitated direct cellular attachment to the treated substrate and enabled multiple points of attachment for neuronal projections. Neurite outgrowth was more easily imaged without the underlying cells, and staining was unnecessary. Common protocols for culturing neural cells which currently require an initial monolayer of cells may be simplified using the PEMs treatment described here.

Supporting Information Available: Static contact angle measurements on variously treated surfaces (Table SI1). AFM analysis of PDMS with micropatterned PEMs surface treatment (e.g., Figures 5 and 6) prior to cell attachment or exposure to cell growth media (Figure SI1). Experimental procedures. This material is available free of charge via the Internet at http://pubs.acs.org.

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