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Hybrid Cell Adhesive Material for Instant Dielectrophoretic Cell Trapping and Long-Term Cell Function Assessment

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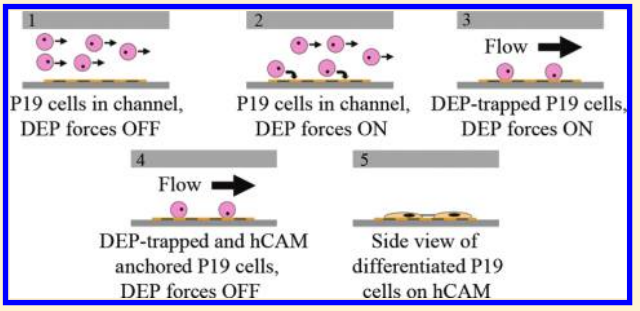
Supporting Information

ABSTRACT: Dielectrophoresis (DEP) for cell manipulation has focused, for the most part, on approaches for separation/enrichment of cells of interest. Advancements in cell positioning and immobilization onto substrates for cell culture, either as single cells or as cell aggregates, has benefited from the intensified research efforts in DEP (electrokinetic) manipulation. However, there has yet to be a DEP approach that provides the conditions for cell manipulation while promoting cell function processes such as cell differentiation. Here we present the first demonstration of a system that combines DEP with a hybrid cell adhesive material (hCAM) to allow for cell entrapment and cell function, as demonstrated by cell differentiation into neuronlike cells (NLCs). The hCAM, comprised of polyelectrolytes and fibronectin, was engineered to function as an instantaneous cell adhesive surface after DEP manipulation and to support long-term cell function (cell proliferation, induction, and differentiation). Pluripotent P19 mouse embryonal carcinoma cells flowing within a microchannel were attracted to the DEP electrode surface and remained adhered onto the hCAM coating under a fluid flow field after the DEP forces were removed. Cells remained viable after DEP manipulation for up to 8 d, during which time the P19 cells were induced to differentiate into NLCs. This approach could have further applications in areas such as cell−cell communication, three-dimensional cell aggregates to create cell microenvironments, and cell cocultures.



INTRODUCTION

The integration of tools for manipulating and controlling cells within microfluidic systems has steadily grown due to the unique features microscale technologies have to offer in terms of fine control over cellular microenvironments, flow conditions, and precise cell positioning for specific cell−cell interactions.[1−4] The combination of such tools with microsystems has enabled the study of cellular processes that otherwise would not have been possible.[5−7] Among the tools currently available to position cells in precise locations on a substrate is dielectrophoresis (DEP), an electrokinetic technique that can trap particles (e.g., cells) on the basis of polarizability differences between the particle and the media in which the particles are suspended when both are exposed to a nonuniform field. The use of DEP has been limited to, for the most part, short-term manipulation studies of cells or preparative methods to separate cells from complex mixtures.[8−11] Few studies have demonstrated DEP trapping for long-term cell experiments where cell function still remains days after the trapping is effected.[1,3] Therefore, it is of paramount importance, when developing DEP devices for in vitro cell studies, to demonstrate that cell viability and cell function (e.g., proliferation, motility, differentiation) are maintained after the electrokinetic manipulation.

A typical design for using DEP to trap cells is the placement of DEP electrodes under a fluid flow in a microfluidic device. This arrangement allows for increased trapping of cells in a short time and the removal of untrapped cells from non-DEP parts of a substrate surface. A challenge to this design is retaining the trapped cells in a fluid flow field at the selected positions when the DEP forces are removed. To produce DEP forces capable of moving cells up the field gradient, known as positive DEP (pDEP), cells must be suspended in sucrose or other low-conductivity media. As opposed to cells suspended in high-conductivity media (e.g., cell growth media), pDEP conditions produce stronger traps, thus attracting more cells and holding them on the substrate while the DEP forces remain active. The difficulty with this arrangement occurs when the DEP forces are switched off and the fluid flow field dislodges the positioned cells. To maintain the cells in position, one needs to have good control over flow so that cells may attach through their integrins or other adhesive proteins over a period of time. An alternative to controlling the flow by pumps and valve systems is to have a

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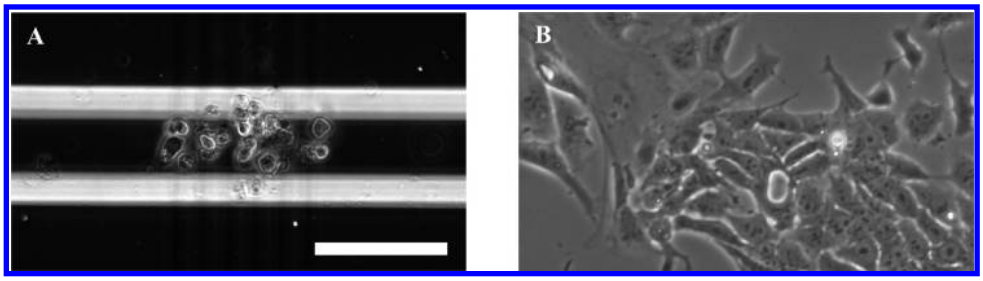

Figure 1. P19 cells attached onto PEMs under different conditions. (A) P19 cells 24 h after DEP trapping in a microfluidic channel. Cells were anchored on PEMs while in sucrose media, and then the sucrose was replaced with cell growth media. Vertical lines in the center are the ITO electrodes used for DEP trapping. (B) P19 cells 24 h after being seeded onto PEMs in cell growth media. Note the difference between P19 cells poorly attached (A) and well-attached healthy cells (B). Scale bar 100 μm.

"sticky" surface to which cells will anchor immediately after DEP trapping is achieved. By taking advantage of the extracellular molecules around the cells, such as antibodies or glycoproteins, either specific or nonspecific binding can be effected. In turn, this can produce cell attachment on the pretreated surface via antibody/antigen binding or electrostatic interactions. The latter approach has been investigated by our group using polyelectrolyte multilayers (PEMs) as the surface coating material and has been shown to work when anchoring cells for short-term experiments.[12] However, a more relevant material for in vitro long-term cell experiments would not only facilitate cell anchoring, but also maintain cell proliferation and cell function.

We have demonstrated cell patterning using PEMs when seeded in cell culture medium[13,14] as well as when trapped under DEP conditions.[12] Cells trapped under DEP and PEM conditions showed that over 93% of them remained anchored on the PEMs after the electrodes were de-energized. However, we have conducted further research in an attempt to extend the use of this approach for long-term cell experiments. The results obtained using PEMs and DEP conditions show deleterious effects on the cells 24 h after DEP cell trapping (Figure 1). Therefore, an alternative to this approach is needed to achieve long-term cell experiments using a sticky surface and DEP. In this paper, we assessed the effects of different cell adhesive materials on the attachment and function of P19 cells to determine the most appropriate surface on which to investigate cell function (specifically differentiation) after DEP trapping and subsequent removal of the electric field. P19 cells are a pluripotent cell line that have the ability to differentiate through several pathways in vitro, specifically neuronal, cardiac muscle, and skeletal muscle. The ability of P19 cells to differentiate after DEP manipulation would demonstrate the successful generation of a cell adhesive material that allows long-term culture. This is critical for performing experiments with cells that are arranged by DEP. Our work demonstrates that an hCAM prepared from fibronectin (FN) and a poly(allylamine hydrochloride) (PAH) layer on top of PEMs allowed for instantaneous cell anchorage after DEP trapping. Furthermore, long-term cell viability (more than a week) and differentiation were also attained, thus demonstrating the utility of the hCAM for long-term in vitro cell experiments.

EXPERIMENTAL SECTION

Materials. PAH (MW = 70 000), PAH−fluorescein isothiocyanate (FITC), monoclonal antineurofilament antibody produced in mouse, antimouse immunoglobulin (IgG)−FITC, retinoic acid (98%), FN, sucrose, poly-L-lysine, and polystyrene pellets were purchased from Sigma-Aldrich (St. Louis, MO).[15] Poly(styrenesulfonic acid) (PSS; MW = 70 000) was purchased from Polysciences, Inc. (Warrington, PA). Poly(dimethylsiloxane) (PDMS; Sylgard 184) was purchased from Dow Corning (Midland, MI). α minimum essential medium (αMEM) with ribonucleosides and deoxyribonucleosides, calcein AM, ethidium homodimer-1,6-carboxy-X-rhodamine, succinimidyl ester (6-ROX-NHS), and fetal bovine serum (FBS) were obtained from Invitrogen Corp. (Carlsbad, CA). P19 cells, 0.25% trypsin−ethylenediaminetetraacetic acid (EDTA) and calf bovine serum (CBS) were purchased from ATCC (Manassas, VA). Purecol (acidified bovine collagen I) was purchased from Advanced BioMatrix (San Diego, CA). Dubelcco's phosphate-buffered saline (DPBS) and phosphate-buffered saline (PBS) were obtained from Mediatech, Inc. (Hernon,VA). Indium tin oxide (ITO)/glass substrates were purchased from Delta Technologies (Stillwater, MN), and 22 mm × 22 mm no. 1.5 Corning coverslips were obtained from Daigger (Vernon Hills, IL). Electrically conductive adhesive was purchased from Epoxy Technology Inc. (Billerica, MA). Octyldimethylchlorosilane was obtained from Gelest (Morrisville, PA). SU-8 photoresist and developer were obtained from MicroChem Corp. (Newton, MA).

Cell Culture. P19 cells were cultured in αMEM with ribonucleosides and deoxyribonucleosides. The growth medium was supplemented by adding 7.5% bovine calf serum and 2.5% fetal bovine serum (37.5 and 12.5 mL in a total of 500 mL of αMEM, respectively). Growth medium was renewed every 2 d, and cells were subcultured every 2−3 d at a dilution ratio of 1:10. Cells were maintained in a humidified environment with 5% carbon dioxide and a temperature of 37 °C.

Biocompatibility Screening of Cells Seeded in Sucrose on Natural and Synthetic Materials. Coverslips were cleaned with isopropyl alcohol (IPA) using a lint-free cloth wipe and were blown dry with compressed nitrogen (N_2) before being placed flat on a glass Petri dish. An 8 mg/mL polystyrene (PS) solution prepared in toluene was spin coated (418.9 rad/s, 50 s) onto the coverslips, and the PS-spin-coated coverslips (PS thickness between 55 and 85 nm) were placed in a vacuum chamber for 3 h to remove any residual solvent. All PS-coated coverslips were plasma oxidized prior to cell adhesive material deposition.

Cells were seeded in sucrose and in cell culture media (CCM) on coverslips coated with natural or synthetic materials. Incubation times were different for each material and pretreatment. Coverslips pretreated with CCM prior to cell seeding were incubated with the CCM for 1 h at 37 °C. Additional coverslips were incubated with collagen I (Col I; 30 μg/mL), poly-L-lysine (1 mg/mL), and FN (25−50 μg/mL) for 90 min at 4 °C. PAH and PSS solutions (1 mg/mL, mol/L (M), concentrations of the repeating units: PAH = 10.7 mmol/L and PSS = 4.8 mmol-L) were each prepared in 18.2 MΩ filtered deionized water (DI-water). The pH of the PAH and PSS solutions was adjusted to 5 and 6, respectively. Four alternating PEMs, $(PAH/PSS)_2$, were deposited onto the oxidized PS surface of the coverslips by immersing the coverslip in the polyelectrolyte solutions sequentially. The initial PAH layer was

deposited for 40 min. The coverslip surface was rinsed with DI-water twice before application of subsequent alternating layers for 10 min with two DI-water rinses between each incubation. After the fourth layer was deposited, the PAH outermost layer (fifth layer) was deposited for at least 30 min at room temperature (approximately 21 ± 2 °C). P19 cells were then seeded in a 0.32 mol/L sucrose solution for 15 min at room temperature, and then the sucrose was aspirated and CCM was added to the cells. Images at 0 h (after addition of CCM at the end of sucrose exposure) and 24 h were taken to assess the morphology differences and adherent status of the seeded cells. The number of cells adhered to the substrates and the number of rounded (i.e., unhealthy) cells were determined with ImageJ software, and the surface that had the highest number of cells with the lowest number of rounded cells was selected for use in the DEP device.

hCAM Surface Preparation. Coverslips for the deposition of the hCAM were prepared using the same procedure as above (see the previous section), but to promote better adhesion of the spin-coated polystyrene, the following silanization step was added prior to spin coating the polystyrene. Cleaned coverslips in a Petri dish were placed in a desiccating chamber containing a Teflon holder with 200 μL of octyldimethylchlorosilane. House vacuum was applied to the chamber for 2 h, and then the Petri dish was placed in a 60 °C oven for at least 3 h. All PS-coated coverslips were plasma oxidized prior to PEM deposition. PEMs were deposited as described in the previous section, except that, after the fourth layer was deposited, the wells were rinsed twice and then stored overnight with DI-water at room temperature.

The PEM-coated coverslips were then incubated in a 50 μg/mL solution of FN prepared in DPBS at 4 °C for 1.5 h. The coverslips were rinsed twice with PBS, and the final hCAM layer was deposited by incubating the coverslips in 1 mg/mL PAH for 45 min at 4 °C. The hCAM coverslips were rinsed twice with DI-water and then transferred to PBS in a new well in a six-well cell culture plate until cell seeding.

The hCAM was deposited onto the ITO electrode substrates as described above, except it was applied in a microfluidic PDMS channel covering the DEP electrodes. In this case, the solutions were added to the channel inlet and flowed down the channel previously aligned onto the DEP electrodes. Once each deposition was completed, the solutions were aspirated via the channel outlet. The incubation times and the concentration of the solutions remained the same.

Cell Seeding on hCAM in Sucrose. A 0.32 mol/L (M) sucrose solution was prepared in DI-water to mimic the osmolarity of the P19 cell culture media but with low electrolyte concentration to maximize DEP forces. Confluent (80%) P19 cells were trypsinized with 0.25% trypsin−EDTA and were divided into two 15 mL centrifuge tubes. The cells were centrifuged for 7 min at 83.8 rad/s and 5 °C. At this point the cells were ready for incubation with sucrose at different time points (0, 15, 30, 45, and 60 min). For the 0 min sample, one tube of cells was resuspended in cell culture media, and the cells were seeded onto the hCAM coverslips at a dilution ratio of 1:10 (~4700 cells/cm^2). The second tube of cells was resuspended in the sucrose solution, and the cells were seeded onto the same substrate at an identical cell seeding density (~4700 cells/cm^2). After each sucrose incubation time point, 4 mL of cell growth media was added to the samples to dilute the sucrose (a 1:27 dilution, 3.7% final sucrose solution) and restore to normal cell culture conditions. Phase contrast images of the P19 cell growth on the hCAM were taken at 0, 24, and 48 h.

Cell Viability on the hCAM Surface. P19 cell viability on the hCAM surface was assessed after 48 h using the LIVE/DEAD viability assay kit from Invitrogen Corp. Calcein AM (excitation/emission maxima at 495 nm/515 nm) was used to stain the viable cells, which exhibit intracellular esterase activity, while ethidium homodimer-1 (EthD-1) (excitation/emission maxima at 495 nm/635 nm) was used to label dead cells with damaged plasma membranes.

Calcein AM and EthD-1 were diluted to 2 and 4 μmol/L, respectively, in a single solution in DPBS. A 1 mL volume of the dye solution was added to each well, and the six-well plates were placed in the incubator for 45 min. The cells were imaged immediately using phase contrast optics and FITC and rhodamine filter sets. The images were taken in triplicate for each time point. Viable and dead cells were counted manually, and the percentage of each was expressed on the basis of the total number of cells per frame. A minimum of 440 cells, per frame, were counted.

Fabrication of the SU-8 Master and PDMS Microfluidic Channels. SU-8 masters with raised features (30−35 μm height, 1000 μm wide) for molding PDMS microchannels were fabricated using the manufacturer's protocol. PDMS microfluidic structures were molded by pouring the polymer on the SU-8 master and curing at 100 °C for 1 h (from the manufacturer's product information sheet).

Fabrication of Patterned ITO Electrodes. ITO electrodes were made from ITO/glass substrates. The ITO surface was patterned using conventional photolithographic methods. A negative photoresist was spin coated onto the ITO surface and then exposed to UV light through a chrome mask containing the electrode design. The pattern was developed, and the exposed ITO was etched using a 9 mol/L (M) hydrochloric acid (HCl) solution. The remaining ITO pattern was then exposed by dissolving the remaining photoresist on the substrate. Wire connections were made by bonding silver/copper wires to ITO pads using an electrically conductive adhesive (H37-MPT, Epoxy Technology, Inc.) heated at 150 °C for 1 h (from the manufacturer's product data sheet).

DEP Cell Experiments. P19 cells were detached from the cell culture surface by trypsinization, centrifuged at 83.8 rad/s for 7 min at 5 °C, and then resuspended in 0.32 mol/L sucrose. The cells were immediately introduced into the microfluidic channel covering the electrodes via the inlet reservoir. Approximately 150 μL of the cells resuspended (~375 000 cells) in sucrose were added to the inlet that accessed the channel previously filled with the sucrose solution. A flow was produced when the cells were introduced due to the difference in pressure between the inlet and outlet reservoirs. Once the cells started to flow down the channel, a sine wave of up to 7 V_{p-p} was applied at a frequency of 30 MHz. The cells were exposed to the DEP forces for up to 4 min, at which point the DEP electrodes were de-energized. Then the cells/sucrose solution in the inlet reservoir was exchanged for cell culture media to replace the sucrose in the channel. The DEP device with the trapped cells in cell culture media was then placed in the incubator set at 37 °C and 5% CO_2.

The cells were maintained by adding fresh cell culture media to the inlet reservoir every 24 h and by removing the media collected in the outlet or waste reservoir at the same time. Images of the cells were taken every 24 h.

Cell Induction and Differentiation. P19 cells are typically induced in suspension. However, our approach requires the induction procedure to be carried out on a surface (hCAM) rather than in suspension. Therefore, we first induced P19 cells on tissue culture polystyerene (TCPS) to determine if it was feasible to induce them on a surface, and then we induced the cells on $(PAH/PSS)_2$/FN and hCAM and compared the results on the three surfaces. Induction on all surfaces was carried out using the same conditions in terms of chemicals and days of induction. The only difference was the surface onto which the P19 cells were attached. The procedure that follows applied to all inductions and was adapted from previously published work.[16] To induce the differentiation of P19 cells, the CCM was replaced by induction media (IM) comprised of αMEM supplemented with 5% FBS and retinoic acid at a final concentration of 0.5 μmol/L. IM was changed every 24 h for a period of 4 d. On day 4, the IM was replaced with CCM, which in turn was replaced every 24 h for 2 d. Cell differentiation was verified by using a fluorescently labeled antibody to stain for marker proteins associated with neuronal differentiation 2 d after cell induction was completed.

Immunocytochemistry. Differentiated P19 cells were fixed by first rinsing the cells with PBS and then adding 4% paraformaldehyde in PBS. The fixation was allowed to occur at room temperature for 10 min, at which time the cells were rinsed with PBS. Cells were then permeabilized with a solution of 0.25% Triton X-100 in PBS and then incubated with the primary antibody (monoclonal antineurofilament) at a dilution of 1:40 for 3 h at room temperature. The samples were rinsed with PBS and incubated at room temperature with a secondary antibody (antimouse IgG−FITC, catalog no. F9137, Sigma-Aldrich) at a dilution of 1:200 for up to 90 min. The neurofilament staining was observed with a 200 M Zeiss microscope using a mercury lamp source and a filter set with a band-pass for excitation at 450−490 nm, a dichroic beam splitter at 510 nm, and a band-pass for emission at 515−565 nm. The objective used had a 10× magnification and an aperture of 0.3. Images were taken using a Zeiss MRm camera.

RESULTS AND DISCUSSION

We carried out two sets of separate experiments to identify a biocompatible coating that allows P19 cell adhesion and growth under low-conductivity sucrose media and under electric fields. TCPS, PS (spin-coated plasma-oxidized), CCM-pretreated PS, poly-L-lysine, $(PAH/PSS)_2PAH$, Col I, and FN were tested as adhesion substrates for cells suspended in sucrose for 15 min, the maximum time we require for DEP positioning of cells. Our assessments (see the Supporting Information) were based on counting the number of adhered cells remaining on the surface and counting the fraction of rounded cells (quantitative cell morphology assessment) as well as qualitative evaluation of cell morphology versus the morphology of the cells on the TCPS substrate 24 h after cell seeding. Previously, we found that PEMs were able to capture cells trapped with DEP forces, but we did not test the compatibility of PEMs for long-term cell culture.[12] Our data indicated (Figure SI1, Supporting Information) that the cells adhered well to the $(PAH/PSS)_2PAH$ (labeled in Figure SI1 as PAH) when the cells were seeded in cell culture media (700 cells/mm^2), but when the cells were seeded in sucrose, fewer than 9 cells/mm^2 were observed. Cell seeding in sucrose significantly decreased the number of adhered cells on collagen, PAH, spin-coated PS with plasma treatment, and fibronectin. Sucrose did not appear to influence adhesion on polylysine and TCPS surfaces. Because we found that sucrose did not appear to decrease cell function when used in tissue culture polystyrene, we hypothesize that sucrose may decrease the adhesive nature of the substrate by blocking or removing the proadhesive molecules. During the substrate evaluation, we noted that although cells remain adhered to collagen I and polylysine after 24 h, greater than 40% and 80% of the cells are rounded and appear unhealthy (Figure SI2, Supporting Information). Representative images of the cells on the different substrates are shown in Figure SI2. Qualitative evaluation of the morphology suggested that the P19 cells that remained adhered to the FN substrate had a spread appearance similar to that of the cells on the TCPS dish (Figure SI3a,b, Supporting Information). Overall, the data from this experiment suggested that the FN substrate best supports growth of the P19 cells when they are seeded from a sucrose solution.

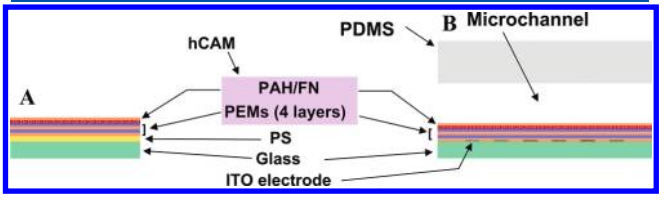

Figure 2. Side view of a glass coverslip (A) and a DEP device (B), both with the hCAM deposited on top. The hCAM is comprised of a layer of FN and PAH on top of four layers of polyelectrolytes ($(PAH/PSS)_2$), which in turn were deposited onto polystyrene-coated coverslips (A) and ITO electrodes (B). The microchannel is molded in PDMS and reversibly bound onto the device.

Experiments in our laboratory showed that when trapping P19 cells under a continuous flow field using DEP and FN-coated substrates, all the cells detached at the moment the DEP forces were stopped (data not shown). To take advantage of the ability of FN to promote long-term adhesion and growth of the P19 cells and the PEMs that support capture of cells after DEP forces are applied, we prepared an hCAM from FN, PEMs, and a PAH layer on top of the FN. A schematic of the hybrid material is shown in Figure 2. The combination of FN adsorbed to PEMs (negatively charged PSS as the outermost layer) and PAH on top of the FN was tested for cell adhesiveness under DEP conditions and long-term cell viability. The cell adhesiveness of the hCAM was assessed first under cell seeding conditions in sucrose. Silanized coverslips were polystyrene coated and then plasma oxidized before deposition of the layers of the polyelectrolytes (see Figure 2A). The procedure to deposit the hCAM was similar for the experiments carried out on the DEP electrodes (see Figure 2B), differing only in the PEMs being directly deposited on the ITO/glass surface and not on a polystyrene layer.

The hCAM was examined by depositing fluorescently labeled FN and PAH in microfluidic channels and imaging the channels using fluorescence microscopy (Figure 3). Fluorescently labeled FN (FN−ROX) and PAH (PAH−FITC) were deposited separately (parts A and B of Figure 3) and then together with PAH−FITC on top of FN−ROX (2C), and in all cases they were deposited on top of four layers of polyelectrolytes ($(PAH/PSS)_2$). All the images in Figure 3 were taken after the channels were rinsed and then refilled with PBS. The fluorescence from

Figure 3. Images of fluorescently labeled components of the hCAM. (A) PAH−FITC and (B) FN−ROX deposited on four layers of polyelectrolytes ($(PAH/PSS)_2$). (C) PAH−FITC deposited on FN−ROX and both on $(PAH/PSS)_2$. The color in (C) denotes the overlapping of the labeled PAH and FN throughout the surface. Scale bar 200 μm.

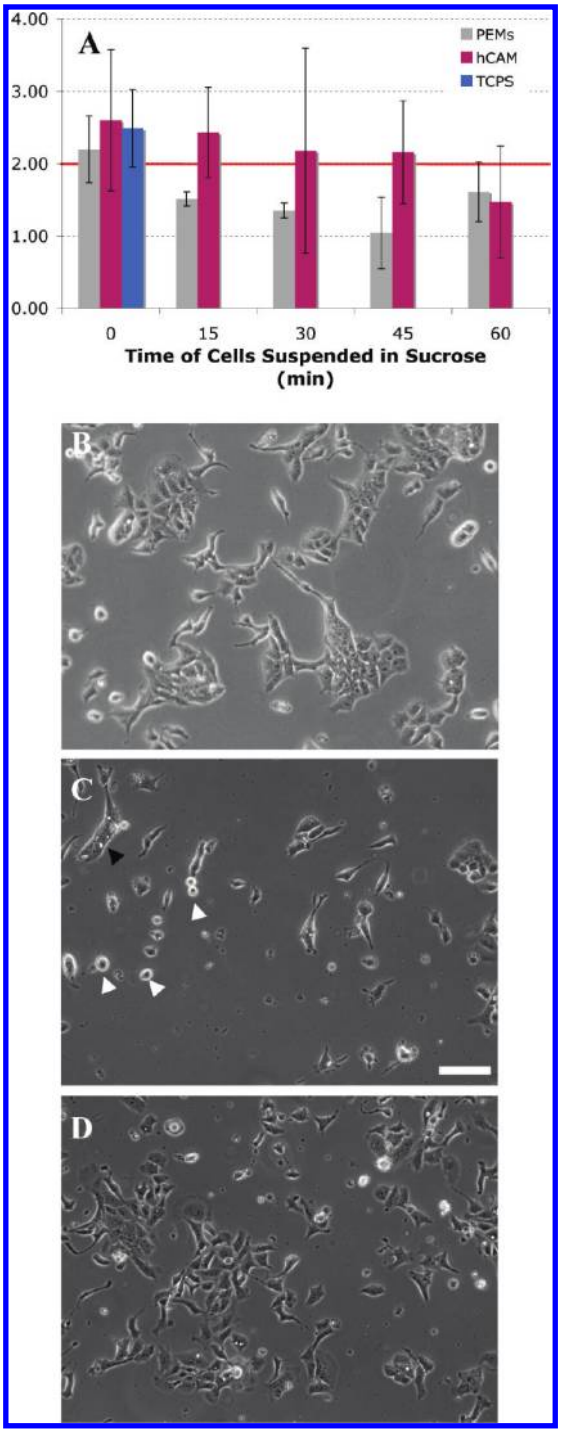

Figure 4. Proliferation of P19 cells seeded onto PEMs and hCAM after resuspension in sucrose. (A) shows the change in the number of cells seeded in sucrose media after 24 h (doubling value). Values are approximately 2 for cells on the hCAM surface, whereas cells on PEMs show values of less than 2 when cells were suspended in sucrose (averages of 89, 44, and 25 cells/frame were observed for TCPS, PEMs, and hCAM, respectively; error bars represent 1 standard deviation). Representative phase contrast images of P19 cells on TCPS (B), PEMs (C), and hCAM (D) 24 h after seeding. Cells on PEMs and hCAM were suspended in sucrose for 15 min. The black arrowhead in (C) indicates a cell with a larger than average surface area, and white arrowheads indicate weak cells attached to the surface of the PEMs. Scale bar 100 μm.

PAH−FITC on $(PAH/PSS)_2$ is shown in Figure 3A. The green color denotes that PAH−FITC homogenously coats the surface. Figure 3B shows FN−ROX bound to $(PAH/PSS)_2$. The red color denotes the FN−ROX, which also covers the surface of the channel. Figure 3C shows PAH−FITC deposited onto FN−ROX, which was first deposited on $(PAH/PSS)_2$. The orange-yellow color indicates the overlapping of the two layers. The orange color is observed on the areas where there is a thin layer of the materials, whereas the yellow areas are observed at the edges of the channel where we have previously observed more accumulation of the deposited PEMs.[17] The PBS rinse was performed by aspirating from the outlet reservoir using a vacuum pump. The fluorescence intensities remained constant after rinsing, suggesting that the hybrid layer is stable in a fluid flow field.

We tested proliferation and viability of P19 cells seeded in sucrose on the hCAM surface. Figure 4A shows the change in the number of cells seeded in sucrose media after 24 h. The cells were exposed to sucrose media for 0, 15, 30, 45, and 60 min, after which CCM was added to the well to substitute the sucrose media. This plot shows a tendency of the hCAM to allow for similar levels of cell proliferation, specifically cell doubling (see the red line in Figure 4A), at all time points. The doubling value was calculated by dividing the number of cells at 24 h by the number of cells seeded at 0 h. A value of 2 is expected if the number of cells doubled. Cells exposed to CCM only (0 min in sucrose) and sucrose for 15, 30, and 45 min showed the best results for the hCAM surface. Only the 60 min sample on the hCAM showed a value of less than 2. On the other hand, the cells seeded on PEMs do not exhibit cell doubling except for those that were seeded in CCM (0 min in sucrose). The average doubling values obtained for P19 cells seeded in sucrose on the hCAM and on PEMs were 2.06 $\pm$ 0.41 and 1.38 $\pm$ 0.25, respectively. These results demonstrate the compatibility of the hCAM with DEP conditions (sucrose media), which is critical to successfully generate DEP trapping forces that will hold cells in place. The PEM alone, on the other hand, was incapable of promoting P19 cell attachment and proliferation (cell doubling) when cells were suspended in sucrose.

The P19 cell morphology after 24 h was also evaluated, where parts B−D of Figures 4 show P19 cells 24 h after seeding on TCPS, PEMs, and hCAM, respectively. These images show P19 cells that were not exposed to sucrose (B) and cells that were exposed to sucrose (C, D) for 15 min and later replaced with CCM. The morphology of the P19 cells was affected by sucrose exposure and the surface on which they were plated. Cells on PEMs appeared more rounded, indicating weak attachment to the surface of the PEMs (see the white arrowheads in Figure 4C). In some cases they formed elongated structures larger than the average surface area of the cells (see the black arrowhead in Figure 4C). Conversely, P19 cells on hCAM showed morphology similar to that of the cells seeded in CCM on TCPS and similar doubling values (doubling value 2.5 on TCPS versus 2.6 on hCAM).

The viability of P19 cells was assessed using a LIVE/DEAD viability assay from Invitrogen Corp. The viability results in Figure 5 show that 99% or more of the cells were viable 48 h after cell seeding on hCAM and 96% of the cells were viable on the PEMs. Also, our results indicate that P19 cells can be exposed to sucrose for at least 60 min with no significant change in viability.

To fully evaluate the function of P19 cells after 15 min of sucrose exposure, we differentiated adhered P19 cells on TCPS

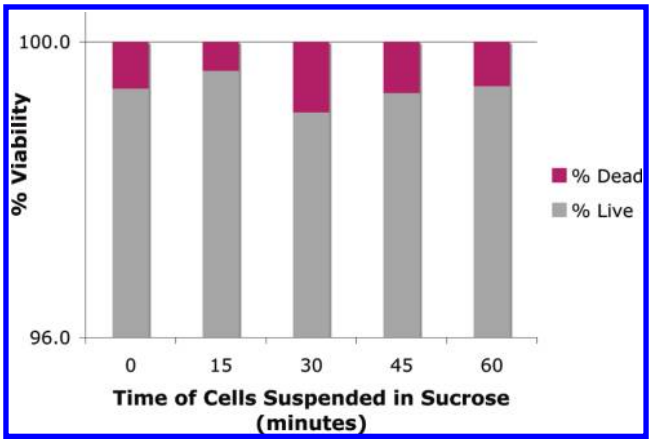

Figure 5. Percent viability of P19 cells on the hCAM. Cells are ≥99% viable at all sucrose exposure times. The percentage of live cells is represented by the gray color bars, whereas the dead cells (red bars) complete the total (100%) of the cells in each bar with ≤1% dead cells.

(control, no sucrose exposure), $(PAH/PSS)_2$/FN, and hCAM. Cell differentiation was evaluated using a procedure that was modified from previous reports on P19 cell differentiation.[16,18] Our process allows for the plating of dissociated cells on adhesive surfaces and induction of differentiation after cell attachment on the substrates. Cell differentiation was carried out by first exposing P19 cells to sucrose for 15 min, exchanging the sucrose for low-serum/retinoic acid induction media, and after 4 d exchanging the low-serum media for normal cell growth media. Figure 6A shows an image of immunostained P19 cells that were induced to differentiate on adhesive TCPS without exposure to sucrose. Neurofilaments and neurofilament proteins in the cytoplasm of the NLCs are stained with a neurofilament antibody.[19] Neurofilaments are observed as cables connecting the cells, and the arrowheads point at neurofilaments generated by the P19 cells, which differentiated into NLCs. Parts B and C of Figure 6 show P19 cells differentiated on $(PAH/PSS)_2$FN and on the hCAM, respectively. Each image shows the clear formation of neurofilaments after P19 cells were induced and differentiated on the surfaces. This indicates P19 cells can be induced to become NLCs and form neurofilaments even when the cells are fully adhered onto these substrates during the programming and induction process.

As previously described, our first attempts to use PEMs for long-term cell experiments demonstrated that polyelectrolytes alone did not maintain cell viability after exposure to DEP conditions (sucrose and electric fields). The hybrid surface we engineered, hCAM, showed the ability to accommodate long-term P19 cell growth and function after the surface and the cells were exposed to sucrose. Once we determined that the hCAM could support long-term cell function, the engineered material was used with a DEP device and all conditions used for such experiments. The combination of polyelectrolytes and FN on the ITO electrodes produced a surface suitable for DEP-based cell anchorage, proliferation, and differentiation as shown in Figures 7 and 8. Figure 7 shows a sequence of cell movement in a fluid flow field and the application of DEP forces. Figure 7A shows a phase contrast image where P19 cells are flowing down the channel (left to right) in the absence of DEP. The cells are passing over the electrodes without being trapped. The first cells trapped when the DEP forces are active are shown in Figure 7B. The applied voltage was varied throughout the experiment from 7 to

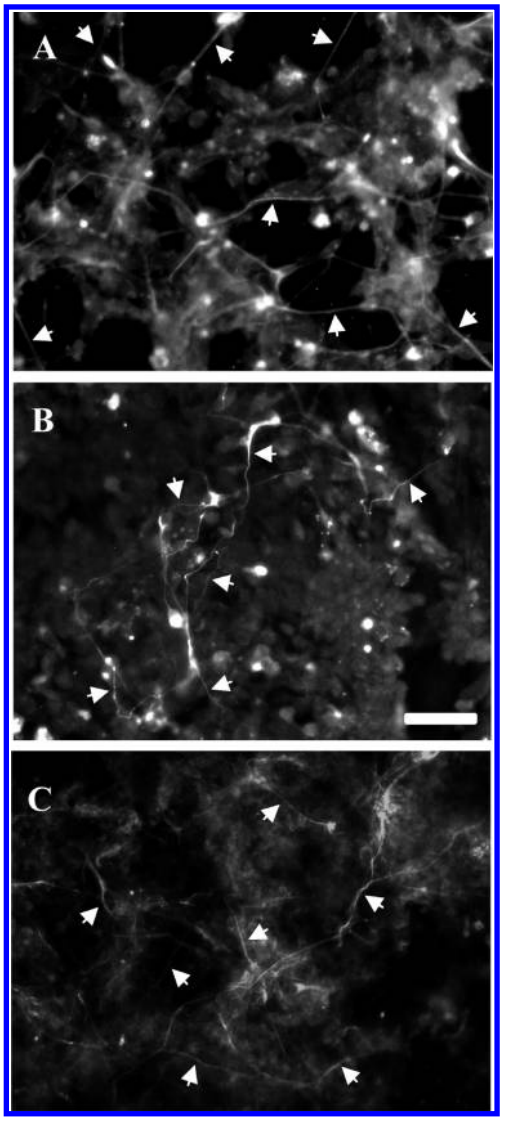

Figure 6. Immunofluorescence images of differentiated P19 cells induced on TCPS (A), $(PAH/PSS)_2$FN (B), and hCAM (C). Neurofilaments were immunostained, demonstrating neuronal differentiation (see the arrows). Induction and differentiation were possible while cells were adhered on all surfaces. Scale bar 50 μm.

3 V at a frequency of 30 MHz (electric fields between 7000 and 3000 V/cm) to start trapping cells on the first pair of electrodes and later cell trappings on subsequent electrodes as the voltage was lowered. Figure 7C shows the P19 cells trapped at the end of the DEP experiment when voltage is no longer being applied. Cells remained trapped under the fluid flow field without an electric field present.

Cells that were trapped under DEP conditions on the hCAM surface in a fluid flow field were later induced to differentiate into NLCs. Figure 8 illustrates P19 cell differentiation after DEP trapping and 8 d in culture in a microfluidic system. The phase contrast image (Figure 8A) shows a number of neurofilament projections connecting the cells once they have differentiated (see the arrowheads), which is indicative of successful P19 cell differentiation into NLCs. The ITO electrodes can be seen (dark gray vertical lines) in the images where cells grew out after initial trapping. Cell migration away from the electrode occurred during

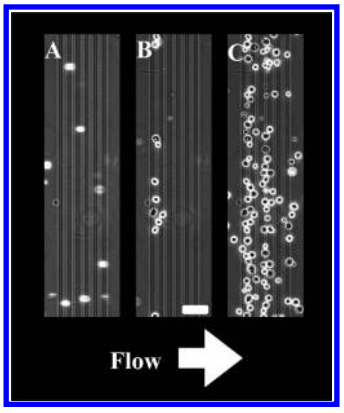

Figure 7. DEP trapping of P19 cells on hCAM. (A) P19 cells flow down the channel, passing over the DEP ITO electrodes (vertical dark gray lines) without being trapped. The ITO electrodes were initially off for a few seconds before they were turned on. (B) Once the electrodes were turned on, P19 cells were trapped by the DEP forces and then anchored onto the hCAM. (C) ITO electrodes were turned off, and P19 cells trapped on the surface remained adhered to the hCAM even while being exposed continuously to a fluid flow field. Scale bar 50 μm.

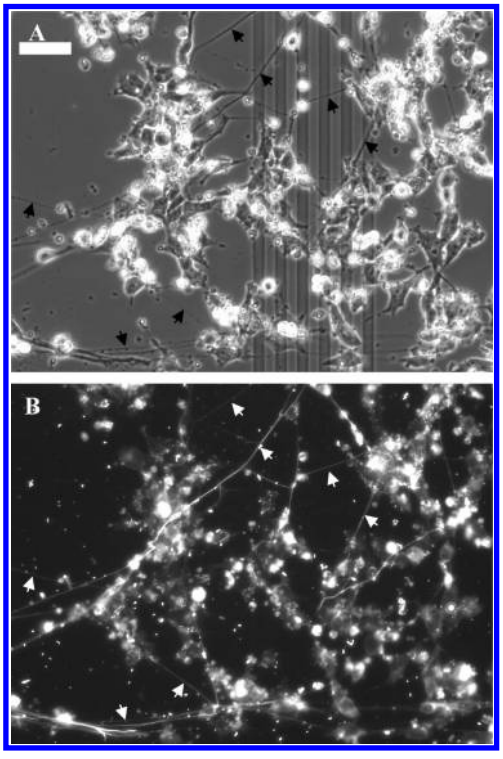

Figure 8. Differentiated P19 cells within a microchannel after DEP trapping and induction. (A) Phase contrast image of NLCs (differentiated P19 cells) on the hCAM after 8 d in the microfluidic system. The vertical dark gray lines are the ITO electrodes used to trap the cells on day 1. Arrowheads point to the projections of differentiated P19 cells. (B) Immunostaining of neurofilaments, a marker of neuronal cells and therefore indicative of successful differentiation of the P19 cells, illustrates the projections from differentiated cells throughout the surface of the device. Cells on the ITO electrodes as well as cells that proliferate away from the electrode regions differentiated equally. Arrowheads point to the neurofilaments formed during cell differentiation. Scale bar 50 μm.

the 6 d (2 d in CCM and 4 d in induction media) required for differentiation.

Immunostaining of neurofilaments better illustrates the complexity of the interconnections among the cells. The fluorescence image in Figure 8B more prominently shows the projections of P19 cells produced after the differentiation process. The presence of stained neurofilament processes and staining in the cytoplasm of NLCs confirms the suitability of the hCAM as a surface that provides for the anchorage of P19 cells under DEP conditions in microfluidics (sucrose media, electric fields, and fluid flow field) and that simultaneously allows the cells to function properly in their ability to differentiate after the complete process.

CONCLUSIONS

In the work presented here, we demonstrated an engineered cell adhesive surface with a two-fold purpose: the anchorage of cells under DEP conditions with a continuous fluid flow field and its ability to support long-term cell experiments such as cell induction and differentiation. We designed a hybrid material comprised of polyelectrolytes and FN, with FN and PAH at the surface that satisfied this goal. The P19 cells were trapped with DEP forces and anchored on the hCAM surface in a continuous flow field within a microfluidic system. The cells were viable for up to 8 d and were able to undergo neuronal differentiation until cell fixation was carried out for immunostaining purposes. We also demonstrated the ability to induce P19 cells while the cells are adhered to a surface. This suggests that neurodevelopment studies that assess cell−cell interactions could be performed in microfluidic devices with hCAM surfaces. Going forward, microfluidics may allow the study of cell-by-cell mechanisms, including the pattern of morphogen response tracked by assessing the fraction of cells that have differentiated. This type of study may be possible by integrating our DEP experimental setup with controlled microfluidic laminar flows.

ASSOCIATED CONTENT

Supporting Information. Figures showing the micrographs of P19 cells 24 h after being seeded onto PS, CCM-pretreated PS, spin-coated PS, PAH on PEMs, poly-L-lysine, fibronectin, and collagen I, total density of P19 cells on test substrates after 24 h in complete CCM, and fraction of rounded cells on test substrates after seeding in 320 mM sucrose. This material is available free of charge via the Internet at http://pubs.acs.org.

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