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Fabrication and Selective Functionalization of Amine-Reactive Polymer Multilayers on Topographically Patterned Microwell Cell Culture Arrays

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Abstract

We report an approach to the fabrication and selective functionalization of amine-reactive polymer multilayers on the surfaces of three-dimensional (3-D) polyurethane-based microwell cell culture arrays. 'Reactive' layer-by-layer assembly of multilayers using branched polyethyleneimine (BPEI) and the azlactone-functionalized polymer poly(2-vinyl-4,4'-dimethylazlactone) (PVDMA) yielded film-coated microwell arrays that could be chemically functionalized post-fabrication by treatment with different amine-functionalized macromolecules or small molecule primary amines. Treatment of film-coated arrays with the small molecule amine D-glucamine resulted in microwell surfaces that resisted the adhesion and proliferation of mammalian fibroblast cells *in vitro*. These and other experiments demonstrated that it was possible to functionalize different structural features of these arrays in a spatially resolved manner to create dual-functionalized substrates (e.g., to create arrays having either (i) azlactone-functionalized wells, with regions between the wells functionalized with glucamine, or (ii) substrates with spatially resolved regions of two different cationic polymers). In particular, spatial control over glucamine functionalization yielded 3-D substrates that could be used to confine cell attachment and growth to microwells for periods of up to 28 days and support the 3-D culture of arrays of cuboidal cell clusters. These approaches to dual functionalization could prove useful for the long-term culture and maintenance of cell types for which the presentation of specific and chemically well-defined 3-D culture environments is required for control over cell growth, differentiation, and other important behaviors. More generally, our approach provides methods for the straightforward chemical functionalization of otherwise unreactive topographically patterned substrates that could prove useful in a range of other fundamental and applied contexts.

Introduction

The collective results of numerous past studies have established the important and diverse roles that surface chemistry and substrate topography can play in directing the functions and fates of cells.[1–13] This base of knowledge has, in turn, motivated the design of new materials and substrates capable of influencing a variety of different cell behaviors, including cell attachment,[2,5,9,14–17] proliferation,[18,19] migration,[1,10,20–22] and, in the case of stem cells,

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Supporting Information Available: AFM image of a film-coated microwell and phase contrast microscopy images of cells attached to film-coated microwells four hours after seeding. This material is available free of charge via the Internet at http://pubs.acs.org.

differentiation to specific cell lineages.[12,23–29] One challenge that must be addressed with respect to the practical utilization of these new research tools, however, is the development of ways to integrate approaches that provide control over individual cell behaviors (e.g., materials that control either attachment or alignment) to design complex materials capable of influencing multiple different behaviors simultaneously (e.g., the design of materials that influence both cell attachment *and* alignment).[8,13,30,31]

The fabrication of topographically patterned surfaces with well-defined and tunable surface chemistry is of interest in a variety of biomedical contexts, ranging from fundamental studies of cell-surface interactions to the design of substrates for tissue engineering and stem cell culture.[4,29,31–34] Efforts to improve control over cell function and design synthetic substrates that mimic the topographic and chemical complexity of *in vivo* cellular environments will ultimately require manipulation and functionalization of materials over a range of length scales. Ideally, processes designed to meet these needs should (i) be low cost and amenable to high throughput fabrication, (ii) permit surface functionalization using a variety of chemical and biological functionality, and (iii) lead to materials platforms that are robust and stable in physiologically relevant environments. In this paper, we present a step toward addressing these broader goals using an approach to the fabrication of covalently crosslinked and chemically reactive polymer films on the surfaces of topographically patterned objects. This approach makes use of methods for the ‘reactive’ layer-by-layer fabrication of azlactone-functionalized polymer multilayers[35–38] to design reactive thin films that permit post-fabrication functionalization and patterning of otherwise inert substrates prepared using conventional lithographic techniques.

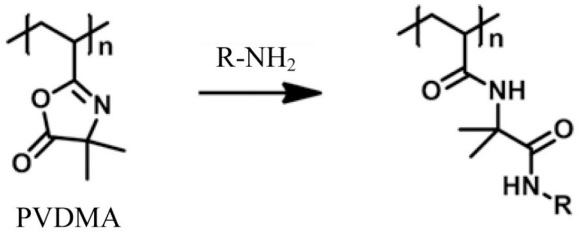
Technological advances for defining the surface chemistry of planar substrates have facilitated spatial patterning of cell adhesion by creating blocks or islands of adhesion-promoting moieties surrounded by passivated, adhesion-resistant backgrounds.[3,7,39–41] While these approaches have been useful for the study of individual cells or monolayers of cells, patterning confined to two dimensions is generally less effective for controlling the proliferation of multilayer cell populations. In these cases, the patterning of three-dimensional (3-D) substrates can provide additional control over cell migration and proliferation.[1,18,20,42–44] One recent approach that shows promise for the study of populations of cells in confined geometries makes use of arrays of micrometer-scale culture wells (so-called ‘microwell arrays’). In this approach, single cells or colonies of cells can be hosted and/or trapped within a well (by virtue of physical boundaries provided by the walls of each well) to add levels of control over cell-cell interactions and help define colony size and shape.[6,34,44–46] In addition, the small sizes of these microwell features allow highly parallel fabrication of high-density arrays of these culture environments.

Numerous lithographic techniques have been used to fabricate topographically patterned surfaces, including microwell arrays, with feature sizes on the order of tens of nanometers to millimeters.[47–49] In particular, the development of approaches to soft lithography[48] has facilitated cost-effective, high throughput methods for substrate fabrication. These methods typically rely on topographically patterned poly(dimethylsiloxane) (PDMS) elastomer stamps molded from a rigid master as a template to transfer an original pattern to a third surface. Of particular relevance to this current study, one specific soft lithography approach (‘replica molding’) uses these PDMS-based stamps as molds for a second pre-polymer, which upon UV or thermal crosslinking creates a polymer-based replica having topographic features similar to those of the original master.[50] Neither of these pattern transfer processes requires a clean room environment or expensive equipment, and both allow the original master and PDMS templates to be reused multiple times.[51]

While these and other lithographic approaches have facilitated topographic patterning of 3-D materials, modification of the surface chemistry of the resulting substrates for use in biological studies remains a significant challenge. Microwell arrays generally require (or at least benefit considerably from) the patterning of surface chemistry to present adhesion-promoting environments in the interiors of the wells and cell-resistant environments in areas outside of the wells.[6,52] However, many polymers used in 3-D replica molding patterning processes, such as polyurethanes, are relatively inert and thereby limit opportunities for post-fabrication surface modification. Past studies have demonstrated approaches to spatially resolved functionalization by (i) physical adsorption of biological or synthetic macromolecules[6,32,52] or (ii) the formation of self-assembled monolayers (SAMs) on thin films of metals (e.g. alkanethiols on gold) deposited onto microwell substrates.[34,46] The first approach takes advantage of non-covalent interactions between macromolecules, such as proteins or polymers, and a surface to facilitate adsorption and chemical modification. For example, Dusselier et al. reported the selective deposition of a PLL-g-PEG graft copolymer outside the wells, followed by the adsorption of fibronectin to the interior surface of the microwells.[52] The second approach permits more controlled and straightforward variation of the compositions of these surfaces using mixtures of alkanethiols during the assembly of SAMs. However, while SAMs provide routes to well-defined and tunable surface functionality useful in a wide range of contexts, issues associated with the long-term stability and biocompatibility of SAMs in physiologically relevant media could limit their use in applications that require the culture of cells for prolonged periods.[53]

The approach to surface functionalization reported here makes use of methods developed recently by our group for the ‘reactive’ layer-by-layer assembly of azlactone-containing polymer multilayers.[35–38] Our past studies have demonstrated (i) that these methods can be used to fabricate ultrathin films (e.g., ranging from 10 nm to several hundred nm thick) on the surfaces of topographically complex substrates, and (ii) that the residual reactive azlactone functionality in these thin films can be exploited to functionalize surfaces and interfaces post-fabrication by treatment with a range of different amine-functionalized molecules (including proteins, peptides, and small molecules that prevent or promote protein adsorption and cell adhesion).[35–38] This current study sought to determine whether this approach to ‘reactive’ assembly could be used to coat the surfaces of polyurethane microwells and differentially pattern surface chemistry to influence the behaviors of cells on these 3-D substrates.

In this study, we report methods for the reactive assembly of thin films on the surfaces of polyurethane microwells by the alternating, layer-by-layer deposition of branched polyethyleneimine (BPEI) and the amine-reactive, azlactone-functionalized polymer poly(2-vinyl-4,4’-dimethylazlactone) (PVDMA; see structure in Equation 1). Characterization using fluorescence microscopy reveals that it is possible to functionalize these films post-fabrication by treatment with amine-functionalized molecules and that this approach permits differential functionalization of the interiors and exteriors of the wells with two different types of functionality. We also demonstrate that these film-coated microwell arrays are useful as substrates for cell culture, and that selective functionalization with structural motifs that either prevent or promote cell adhesion (e.g., arrays of azlactone-functionalized wells, with regions between the wells presenting adhesion-resistant motifs) can be used to confine and control populations of cells within the wells of these arrays for periods of up to four weeks and support the 3-D culture of cubiodal cell clusters.



Eq. 1

Materials and Methods

Materials

2-Vinyl-4,4'-dimethylazlactone (VDMA) was a kind gift from Dr. Steven M. Heilmann (3M Corporation, Minneapolis, MN). Branched polyethyleneimine (BPEI; MW = 25,000), 3-aminopropyltriethoxysilane (APTES), reagent grade DMSO, and acetone were purchased from Aldrich Chemical Company (Milwaukee, WI). Tween 20 surfactant and glass microscope slides were purchased from Fischer Scientific (Pittsburgh, PA). PDMS (Sylgard 184 Silicon Elastomer) was purchased from Ellsworth Adhesives (Germantown, WI). PVDMA (M_w = 20,400; PDI = 3.3) was polymerized from VDMA monomer as described in our previous reports.[37] Fluorescently labeled PVDMA was synthesized from tetramethylrhodamine cadaverine and PVDMA (TMR-PVDMA) as described previously.[37] Labeling of the polymer was ≤ 0.5% based on equivalents of TMR added to the reaction solution. D-Glucamine was purchased from TCI America (Portland, OR). Dulbecco's modified Eagle Medium (DMEM), calcein AM, Hoechst 34580 and wheat germ agglutinin-Alexa 594 (WGA 594) fluorescent cell stains, and tetramethylrhodamine-5-(and-6)-isothiocyanate (TRITC) were purchased from Invitrogen (Carlsbad, CA). Tetramethylrhodamine cadaverine (TMR-cad) and fluorescein-5-isothiocyanate (FITC) were purchased from AnaSpec, Inc. (San Jose, CA). COS-7 and HEK 293T/17 cells were obtained from ATCC (Manassas, VA). All materials were used as received without further purification unless noted otherwise.

General Considerations

Compressed air used to dry films and coated substrates was filtered through a 0.2 μm membrane syringe filter. Atomic force microscopy (AFM) data were acquired in tapping mode on a Nanoscope Multimode atomic force microscope (Veeco/Digital Instruments, Santa Barbara, CA), using scan rates of 20 μm/s to obtain 256 × 256 pixel images. Silicon cantilevers with a spring constant of 40 N/m were used (model NSC15/NoAl, MikroMasch USA, Inc., Portland, OR). Height data were flattened using a 2nd-order fit. The surface roughness was calculated from images using the Nanoscope software (Veeco/Digital Instruments, Santa Barbara, CA). Optical and fluorescence microscopy images were acquired using an Olympus IX70 microscope and analyzed using the Metavue version 4.6 software package (Universal Imaging Corporation). Confocal Microscopy images were taken on a Nikon A1R confocal microscope and processed using NIS-Elements C imaging software.

Replica Molding of Polyurethane-Based Microwell Substrates

Glass microscope slides used for the fabrication of microwells were first coated with an aminosilane coating to improve adhesion of the polyurethane to the glass during long-term cell culture. To this end, APTES was deposited on the glass surface from a 1% solution in

anhydrous toluene over 1 hour, followed by rinsing with toluene and ethanol and annealing at 100 °C for one hour. Polyurethane microwells were fabricated on these treated glass slides as described previously.[34] Briefly, we used photolithography and plasma etching techniques to produce silicon masters with depressions of desired patterns (see text for additional descriptions of dimensions and layout). After passivating the surfaces of the silicon masters, PDMS stamps were manufactured by pouring an elastomer prepolymer and curing agent mixture (10:1 w/w) over the masters and allowing the mixture to polymerize. To form a mold to cast the final microwell substrate, PDMS stamps were clamped to the aminosilane-coated glass microscope slides prepared previously (separated by 250 mm spacers). Norland optical adhesive 61 (Norland Products Inc., Cranbury, NJ, USA) polyurethane prepolymer was fed to one end of the clamped stamps and distributed via capillary action. After crosslinking under UV light for 20 min, stamps and spacers were removed to yield patterned microwells.

Synthesis of Fluorescently Labeled BPEI

The synthesis of fluorescently labeled derivatives of BPEI was performed according to the following general protocol. BPEI (220 mg, 5.12 mmol) was weighed into a 10 mL round-bottomed flask and dissolved in ~1 mL DMSO. For BPEI functionalized with fluorescein (FITC-BPEI), 0.005 equiv. of FITC (9.8 mg, 0.0252 mmol) was dissolved in ~1 mL DMSO and added dropwise. The flask was capped with a septum and stirred in the dark at room temperature for >16 h. The solution was then transferred to dialysis tubing (Spectra/Por Dialysis Membrane, MWCO 3,500) and dialyzed against deionized water for 36 hours, replacing the dialysis solution regularly, to remove unreacted dye. After dialysis the reaction solution was frozen in liquid nitrogen and lyophilized overnight to yield an orange, very viscous liquid. BPEI labeled with TRITC (TMR-BPEI) was prepared by the same protocol, using 0.005 equiv. of (TRITC) (2.6 mg, 0.0059 mmol) dissolved in 400 μL DMSO added to BPEI (51.6 mg, 1.2 mmol) dissolved in 250 μL DMSO. Lyophilization yielded a light pink solid. Labeled BPEI polymers were stored in a vacuum desiccator and used without further purification. On the basis of the method of synthesis described above, these polymers were regarded as being labeled at low mole percentages of ≤0.5% and were sufficient for all subsequent experiments used to characterize the presence or absence of these polymers using fluorescence microscopy.

Layer-by-Layer Fabrication of Polymer Multilayers

Solutions of BPEI and azlactone-containing polymer (either PVDMA or TMR-PVDMA) were prepared in acetone (20 mM with respect to the molecular weight of the polymer repeat unit). Films were deposited layer-by-layer on microwell substrates as follows. First, substrates were immersed in a solution of BPEI for 20 seconds. The substrates were then removed and immersed, with gentle agitation, in a rinse solution of acetone for approximately 5 seconds, followed by a second rinse in a stream of fresh acetone. Substrates were then immersed in a solution of PVDMA for 20 seconds, followed by rinsing in the manner described above. This cycle was repeated until 10 BPEI/PVDMA layer pairs (or ‘bilayers’) were deposited on the surface. Following the final rinse, substrates were dried in a stream of filtered compressed air and stored in a vacuum dessicator at least overnight before use. All film fabrication was carried out at room temperature.

Post-Fabrication Functionalization of Film-Coated Microwells

Film-coated microwells were functionalized after film fabrication using aqueous solutions of various primary amine-containing molecules in a spatially resolved manner (e.g., by deposition of one compound outside the wells and, if desired, a second, different compound inside the wells) using the following general approach. First, a thin film of reaction solution was spread on a flat piece of glass. (For these experiments, solutions of labeled BPEI were

20 mM (with respect to repeat unit) in Milli-Q water, and solutions of glucamine contained 20 mg/ml glucamine in Milli-Q water, with the pH adjusted to 9.75; see text for additional details). A patterned microwell substrate was then turned upside down (inverted) such that the microwells were facing down and gently placed onto the liquid-coated glass. Air bubbles trapped in the wells prevented reaction solution from entering wells (as shown in Scheme 1B). Substrates were left in place for ~25 minutes and then rinsed with deionized water to yield dual-functionalized arrays (e.g., arrays having azlactone-functionalized wells and differentially functionalized areas in between). In some experiments, a second molecule was then reacted inside the wells by placing a large drop of a second reaction solution onto the microwell surface (turned face up; as shown in Scheme 1C). After reacting for ~40 minutes, substrates were rinsed in deionized water and dried in a stream of filtered compressed air. Substrates were used immediately or stored in a vacuum dessicator for later use.

Cell Culture, Staining, and Imaging

Experiments designed to demonstrate spatial control over cell adhesion were performed using film-coated microwell substrates with the surfaces outside of the wells either treated with glucamine or left unreacted (see text and protocol above for details). Substrates were pre-rinsed with ethanol and serum-free Dulbecco's modified Eagle medium (DMEM) to ensure that the microwells were filled with liquid. These substrates were then placed individually into the wells of 6-well tissue culture treated polystyrene culture plates. In all cases, the media used was as follows: 90% DMEM, 10% fetal bovine serum for COS-7 and HEK 293T/17 cells; 100 units/ml penicillin and 100 μg/ml streptomycin were added to media for all cases. All cells were seeded on the substrates at an initial density of 300,000 cells/mL. Approximately 200 μL of seeding suspension was added directly to the tops of the substrates without allowing media to spill off of the substrates. Cells were allowed to settle and attach to the surface for 20 minutes at room temperature, after which an additional 2 mL of cell culture medium was added. Cells were then incubated at 37 °C for at least 24 hours. At predetermined intervals (e.g., either 24 hrs or 14, 21, or 28 days) the media was aspirated and replaced with 2 mL of a calcein AM staining solution (1 μg/mL in PBS) for 30 minutes at 37 °C. Following incubation, the staining solution was aspirated and replaced with 2 mL of cell culture medium. For experiments using HEK 293T/17 cells to characterize the growth of cell clusters at longer time points, cells were stained with a solution of Hoechst 34580 (5 μg/mL) and WGA 594 (18 μg/mL) in PBS for 10 minutes at 37 °C. Cells were imaged by optical light microscopy and fluorescence microscopy without removal of the glass substrates from the culture wells, or by LSCM with the substrates removed and inverted on a glass cover slip. For longer-term experiments, growth medium was aspirated and replaced every 2–3 days as needed during periods when cells were not being stained and imaged.

Results and Discussion

Fabrication and Functionalization of BPEI/PVDMA Films on Polyurethane Microwell Arrays

We have demonstrated in past reports methods for the layer-by-layer assembly of amine-reactive and covalently crosslinked polymer multilayers using BPEI and PVDMA.[35–38] In contrast to methods for the aqueous layer-by-layer assembly of polyelectrolyte-based multilayers, the fabrication of these reactive materials is conducted entirely in organic solvents and is mediated by fast interfacial reactions between the primary amines of BPEI and the amine-reactive azlactone functionality of PVDMA (e.g., see Equation 1). Past studies have demonstrated that these films can be fabricated at a wide range of surfaces, including planar glass and silicon substrates[35,38] and interfaces created between two immiscible liquids,[36] and that these materials can be deposited conformally onto topologically complex substrates, such as fibers[37] and the surfaces of electron microscopy

grids.[38] We have also demonstrated that the residual azlactone functionality in these materials can be used to functionalize the surfaces of film-coated objects by treatment with primary amine-containing molecules.[35–38]

We performed a series of initial studies to determine whether this general approach could be used to fabricate and functionalize thin films on the topographically patterned surfaces of polyurethane-based microwell arrays used previously by our group as 3-D substrates for cell culture.[34,54] For these experiments, we used model microwell substrates containing 25 × 25 arrays of cuboidal wells measuring 300 μm on each side and 120 μm deep fabricated from polyurethane on the surfaces of aminosilane-treated glass microscope slides. Layer-by-layer fabrication of BPEI/PVDMA multilayers was performed directly on these substrates without additional treatment (Scheme 1A; see Materials and Methods for additional details). For these experiments, we deposited 10 layer pairs (or 'bilayers') of BPEI and PVDMA. The results of our past studies demonstrate that 10-bilayer films provide a platform that is sufficiently stable to permit incubation and manipulation for extended periods in cell culture media without significant tearing or degradation.[35] We note that the presence of the underlying elastomeric polyurethane substrate complicated direct characterization of the thicknesses of these films (e.g., by ellipsometry or by using AFM to characterize a scratched film). Our past studies, however, demonstrate that 10-bilayer BPEI/PVDMA films fabricated on planar silicon substrates are ~100 nm thick.[38]

Fabrication of films using tetramethylrhodamine-labeled PVDMA (TMR-PVDMA) and BPEI permitted further characterization of film-coated substrates using laser scanning confocal microscopy (LSCM). Figure 1 shows a cross-sectional fluorescence profile of a single microwell coated with a BPEI/TMR-PVDMA film 10 bilayers thick (this image was generated from a stack of confocal images reconstructed in 3-D and vertically sliced using software). This image reveals the presence of fluorescence on the bottom, the edges, and the corners of the wells, as well as on the top surface of the substrate (i.e., in areas outside of the microwell). On the basis of these results, we conclude that layer-by-layer fabrication was sufficient to deposit uniform and conformal polymer multilayers over the entire surface of these micrometer-scale features. Additional characterization of film-coated substrates using atomic force microscopy (AFM) revealed these surfaces to be smooth (with a rms roughness of ~3 nm; a representative AFM image is included in Figure S1 of the Supporting Information).

We next performed a series of experiments to determine whether the BPEI/PVDMA films on these substrates could be used to functionalize and pattern the surfaces of the microwells post-fabrication. To facilitate spatially resolved patterning of chemical functionality, we developed a straightforward method to functionalize only the top surfaces of the arrays (i.e., the spaces outside of and between the wells) by treatment with water soluble, amine-containing molecules. Our general approach is illustrated in Scheme 1B. Microwell substrates were inverted and placed onto a piece of glass coated with a thin layer of an aqueous solution of amine. This experimental approach had the advantage that air trapped inside the microwells (combined with the relatively hydrophobic nature of untreated BPEI/PVDMA films and the surface tension of water) effectively prevented the aqueous reaction solution from entering the wells and restricted functionalization to areas outside of the wells. We note that several past studies have reported using inverted microcontact printing (i-μCP) using a featureless (i.e., flat) stamp to achieve the same result.[32,33,55,56] For the purposes of this current investigation, however, our approach allowed us to spatially pattern a variety of large and small water-soluble molecules (as described below) without having to optimize flat-stamping reaction conditions for each different type of molecule. Although these alternative methods could also prove useful, our approach was sufficient for all studies described here.

Figure 1B shows a fluorescence microscopy image of a film-coated array functionalized by treatment with a solution of FITC-BPEI for 20 minutes using the method described above (the film was rinsed extensively with deionized water prior to imaging). The image shows clearly the presence of fluorescence in a defined grid pattern, with areas between the wells appearing green and areas within the wells remaining dark. Additional cross-sectional imaging using LSCM revealed the absence of fluorescence on the walls of the wells (data not shown). These patterns of fluorescence demonstrate that this general approach can be used to spatially localize the deposition of FITC-BPEI. These results also suggest that residual azlactone functionality present in these films can be used to functionalize the surfaces of these microwell substrates, consistent with the results of our past studies. We note that this selective functionalization procedure yields dual-functionalized microwell substrates (i.e., the azlactone functionality present on the surfaces of the wells of these treated substrates remains unreacted). We return to a discussion of this residual azlactone functionality again in the discussion below.

Functionalization of Film-Coated Arrays with Molecules that Prevent or Promote Cell Adhesion

Previous reports from our group have demonstrated that it is possible to control the behaviors of cells on two-dimensional BPEI/PVDMA films fabricated on rigid glass substrates by functionalization of the films using primary amine-containing small molecules that either prevent or promote cell adhesion.[35] For example, whereas treatment with the hydrophobic small molecule *n*-decylamine yielded surfaces that promoted the adhesion of fibroblast cells, treatment with the hydrophilic amine-containing carbohydrate D-glucamine prevented cell attachment almost completely. The results of additional experiments suggested that this behavior was facilitated, at least in part, by the ability of these decylamine- and glucamine-functionalized films to either promote or prevent the adsorption of protein, respectively.[38] This past report also demonstrated that azlactone-functionalized (untreated) films promoted significant cell attachment and growth, presumably by the deposition of serum proteins, which can react with azlactone functionality via surface-accessible lysine residues,[57–59] on the surfaces of these films.

We performed a series of experiments to (i) determine whether treatment with glucamine could be used to localize the adhesion of cells exclusively inside the wells of these arrays, and (ii) evaluate the initial effects of long-term culture of cells on these film-coated substrates. For these experiments, we used the approach described above to functionalize the areas outside of the wells of a film-coated array by treatment with a solution of glucamine (20 mg/mL in PBS, pH = 9.75) for 25 minutes. The azlactone-functionalized interiors of the wells were left unreacted prior to cell seeding to allow deposition (and likely covalent immobilization, as described above) of serum proteins from the medium and promote cell adhesion. We then seeded African green monkey kidney fibroblasts (COS-7 cells) directly on the surfaces of these dual-functionalized, film-coated arrays. After 24 hours, cells were stained with calcein AM, a small-molecule stain that fluoresces when cleaved by esterases in viable cells, to provide a measure of cell viability and assist with characterization of the locations and morphologies of cells in these experiments. Figures 2A and B show representative fluorescence microscopy and phase contrast images, respectively, of a patterned microwell substrate. The image in Figure 2A reveals cells to be localized inside the microwells; very little to no cell-associated fluorescence was observed between the wells. These results demonstrate that the cells remain viable and that they only attach and grow in the untreated areas located inside the wells. Images of cell-seeded substrates acquired at earlier time points (e.g., 4 hrs) demonstrated that while cells do initially attach and adopt a spread morphology in azlactone-functionalized wells after seeding, they do not attach and spread in glucamine-treated regions at these early times. These results

demonstrate that the spatial patterns observed in Figure 2 (e.g., acquired at 24 hours) result from the ability of glucamine-treated regions to prevent the initial adhesion of cells (i.e., that that the absence of cells in these regions in Figure 2 does not result from initial attachment followed by cell detachment or death). Images of cell-seeded substrates acquired at time points earlier than 24 hours are included in Figure S2 of the Supporting Information.

Figure 2B shows a representative phase contrast image, and confirms the lack of cells between wells (additional treatment of these substrates with ethidium homodimer, a fluorophore-based dead cell stain also revealed the absence of dead cells in these regions; data not shown). This phase contrast image also reveals the formation of small bubbles between the polyurethane film and the glass substrate. These bubbles appeared after several hours in culture but did not appear to affect the stability of the polyurethane arrays or significantly alter cell behavior. The image in Figure 2C shows a lower magnification image of the same array, and demonstrates that the patterning of cells within wells is maintained with fidelity over large areas of the substrate (e.g., over areas as large as 1.5 cm^2, or areas encompassing at least 400 microwells) with few visible defects. Figure 2D presents a higher magnification fluorescence microscopy image of a single well. This image reveals the cells in this well to be nearly confluent and demonstrates more clearly the extent to which cells are able to attach and adopt a spread morphology inside these wells. These results are generally consistent with those of previous experiments in which COS-7 cells were cultured on unmodified BPEI/PVDMA films in serum-containing media.[35] Finally, the image in Figure 2E shows results obtained using a control substrate that was not reacted with glucamine at all prior to cell seeding. This image reveals that cells attach and spread in the unmodified areas between the wells. This result demonstrates that the absence of cells in these same areas in Figures 2A,C, and D is a result of the glucamine treatment of those substrates, and not simply a result of the topographic features of the microwell substrates.

Returning to the higher magnification image shown in Figure 2D, we note the presence of brighter fluorescence around the inside edges of the well (this increase in intensity is also evident, albeit less clearly, in the lower magnification image in Figure 2A). Characterization by LSCM reveals this brighter fluorescence to arise from the presence of cells on the vertical edges of these wells. Figure 3 shows a series of x–y scans collected beginning with the focal plane at the bottom of the well (A) and then acquiring images at approximately 45 μm (B) and 90 μm (C) above the bottom of the well. Figure 3A shows a nearly confluent layer of cells across the bottom of the well, consistent with the high magnification image in Figure 2D. Figure 3B reveals faint fluorescence in the center of the well, likely arising from cells attached to the bottom of the well, as well as several cells attached to the edge of the well. Finally, in Figure 3C, distinct cells were observed on the wall of the well, including cells present in locations that were dark in the image in Figure 3B. These results, when combined, demonstrate that cells are able to grow on the edges of these wells and suggest that our approach to glucamine treatment limits functionalization to areas outside of the wells (that is, that wicking or diffusion of glucamine down into the wells does not occur or, at least, that it does not inhibit the attachment of cells on the walls of the wells significantly).

We performed a second series of cell-based experiments to characterize the long-term stability of our film-coated arrays, both in terms of physical integrity and the ability of glucamine-treated areas to prevent cells from growing out of the wells over time. These experiments were performed using dual-functionalized microwell substrates prepared as described for the short-term experiments above (i.e. glucamine treatment in areas outside of the wells, and azlactone-functionalized surfaces of well interiors left untreated), but cell-seeded arrays were maintained and observed in culture for up to 28 days. For these longer-term experiments, medium was exchanged periodically and cells were stained with calcein AM every seven days to visualize cells and characterize viability. Figures 4A–E show

representative fluorescence microscopy images of cells at various time points during these experiments. Figure 4A shows cells localized to the wells one day after seeding; these results are similar to the image in Figure 2A. Figures 4B and C show images of the same substrate after 14 days and 28 days of continuous culture, respectively. We note that these images do reveal the presence of a few isolated cells in glucamine-treated regions. The locations of these isolated cells suggest, however, that they may have detached from other locations during these experiments and landed in these areas.[35] We note further, however, that we did occasionally observe instances in which cells appeared to be growing and/or moving into glucamine-treated regions of the arrays after longer periods of time (e.g., after 21 days, as shown in Figure 4D). Finally, the image in Figure 4E shows a microwell substrate for which only part of the substrate was functionalized with glucamine prior to cell seeding (the remainder was left unfunctionalized; image acquired 21 days after seeding). Inspection of this image reveals cells to grow on and cover completely the areas of these arrays that were not treated with glucamine over the course of these more extended experiments.

The general lack of cells in the areas between the wells at these extended time points suggests that the films remained intact and that the non-fouling properties imparted by glucamine treatment remained able to inhibit cell attachment significantly (no other visible wrinkling or apparent detachment of the films was observed). These results also demonstrate that the surfaces inside the wells were capable of supporting cell growth and viability for extended periods of time. Additional characterization using LSCM revealed these wells to fill up with cells over time. We note, however, that COS-7 cells are generally contact-inhibited, and therefore do not represent an optimal cell system for the characterization of time-dependent growth of cell multilayers or the ability of these functionalized microwell substrates to promote and confine the 3-D growth of cuboidal cell clusters. We therefore conducted additional long-term culture experiments using these functionalized arrays to characterize the 3-D growth of HEK 293T/17 cells, a cell type that is not contact inhibited. Figure 5 shows a representative confocal microscopy cross-sectional image of a well 3 weeks after the initial seeding of HEK cells (cells in this image are stained with Hoechst nuclear stain and WGA-594 membrane stain. These data reveal the presence of cells growing uniformly across the top, open face of the well, consistent with the growth of a 3-D cell mass that fills the entire well geometry. Closer inspection of this image reveals that cells have not migrated laterally out of the well, suggesting that the presence of glucamine in the regions surrounding the wells is sufficient to prevent outgrowth of HEK cells. These results are consistent with the results of experiments described above for COS-7 cells and demonstrate that the ability of glucamine treatment to prevent cell attachment and migration is not cell-line dependent.

Secondary Functionalization of Azlactone-Functionalized Wells

Finally, we note again that the interior surfaces of the wells used in the experiments described above were not treated with amine functionalized compounds (that is, the azlactone-functionalized surfaces of the wells were not modified chemically prior to the addition of media and cells). The presence of reactive azlactone groups, however, opens the door to further functionalization of the microwell environment to introduce chemical or biological motifs (e.g., cell adhesion peptides or other motifs) that could, ultimately, be used to enhance or direct the behaviors of cells.

We performed an additional series of experiments to determine whether unreacted azlactone functionality on the interior surfaces of the wells could be used to functionalize the wells using a second, different amine-containing molecule. To demonstrate proof of concept for such secondary functionalization, we performed an experiment in which two different fluorescently labeled BPEI derivatives were immobilized on the substrate surface. Using a

two-step procedure (depicted in Scheme 1C), we first reacted the top surface of the array using TMR-BPEI in the manner described above. Following extensive rinsing, the entire microwell substrate was then immersed in a solution of FITC-BPEI for 40 minutes. Because the fluorescence spectra of TMR and FITC are substantially non-overlapping, characterization of these treated substrates using fluorescence microscopy permitted independent visualization of the spatial distributions of these two different polymers. Figure 6A–C shows representative images acquired through the red channel (A), the green channel (B), and a merged image (C) of a substrate treated in this manner. Inspection of these images reveals TMR-BPEI to be present in the areas between the wells, and that FITC-BPEI (used here during the second processing step) is present only in the regions inside the wells. The fact that FITC-BPEI is present only in the areas inside the wells, despite having come into contact with the entire surface of the array when it was submerged during the second processing step, demonstrates that the initial reaction of the outer surface using TMR-BPEI was sufficient to prevent the deposition of this second species on the surface. This observation suggests that initial treatment with TMR-BPEI could be sufficient to react with virtually all remaining azlactone functionality present in these areas. We note, however, that our results do not rule out the possibility that the presence of the TMR-BPEI deposited initially could block any further reaction or adsorption of FITC-BPEI by steric inhibition or by electrostatic repulsion. In any case, the results of this experiment demonstrate that it is possible to use this approach to deposit two different chemical entities on the surfaces of these microwell substrates in a manner that is both spatially resolved and does not lead to any significant overlap. The two different fluorescently labeled cationic polymers used in this proof of concept experiment do not have mutually exclusive biological functions. However, experiments to evaluate the suitability of this secondary functionalization process to differentially deposit different chemical and biological motifs capable of directing the growth and/or differentiation of 3-D cultures of other cell types of biomedical interest (e.g., human pluripotent stem cells) are currently underway.

Summary and Conclusions

We have reported a ‘reactive’ layer-by-layer approach to the fabrication of thin, conformal, and covalently crosslinked polymer multilayers on the surfaces of 3-D polyurethane-based microwell cell culture arrays. Our results demonstrate that film-coated arrays can be functionalized post-fabrication by treatment with either amine-functionalized macromolecules or small-molecule amines to produce dual-functionalized microwell substrates. Treatment of film-coated arrays with the small-molecule amine D-glucamine resulted in surfaces that resist the adhesion of mammalian cells *in vitro*, consistent with the results of past studies of glucamine-functionalized films on planar glass substrates. In addition, glucamine functionalization in areas of the arrays between the wells yielded dual-functionalized array substrates that could be used to confine cell attachment and growth to microwells for periods of up to 28 days. The results of other experiments demonstrated an alternative approach to the patterning of dual functionalized substrates by sequential treatment with two different fluorescently labeled cationic polymers (e.g., functionalization of the surfaces of the wells with one polymer, and the regions between the wells with a second, different polymer). These approaches to dual functionalization could prove useful for the long-term culture and maintenance of cell types (e.g., stem cells) for which the presentation of specific and chemically well-defined 3-D culture environments is believed to be required for control over cell growth, differentiation, and other important behaviors. More generally, this approach to ‘reactive’ layer-by-layer assembly provides new polymer-based methods for the introduction of chemical and biological functionality to otherwise unreactive topographically patterned substrates and could therefore prove useful in a range of other fundamental and applied contexts.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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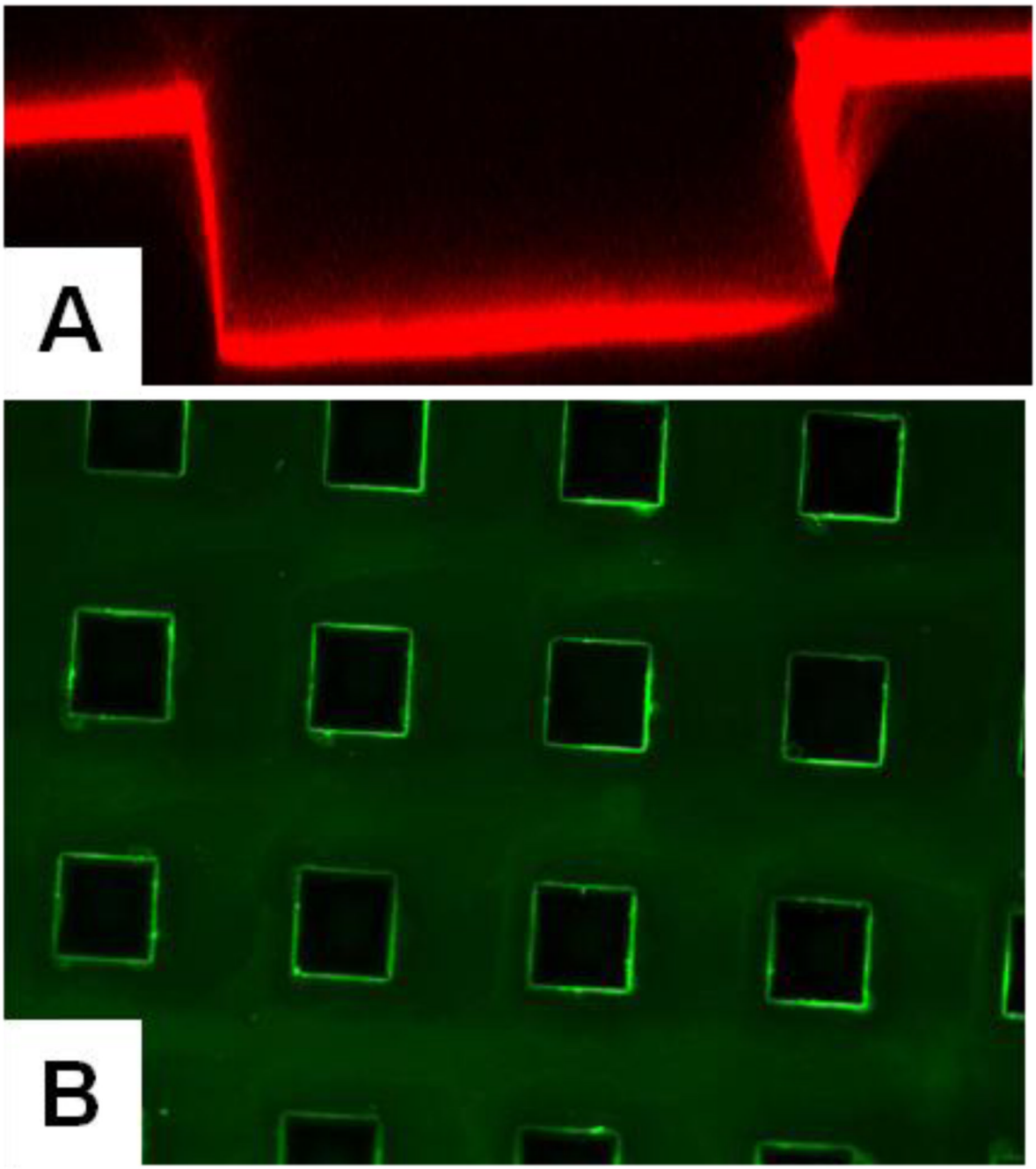


Figure 1.

A) Cross-sectional LSCM image of a single microwell of an array coated with a BPEI/TMR-PVDMA film 10 bilayers thick. The substrate was tilted on the microscope stage to facilitate imaging of the left side and bottom of the well. The dark part of the image near the right side of the well is the result of optical effects that distort the image. B) Representative fluorescence micrograph (4X) of a film-coated microwell substrate patterned by exposure to a solution of FITC-BPEI to functionalize the areas located between wells using the method from Scheme 1B. Microwell dimensions are 300 μm on each side, and 120 μm deep.

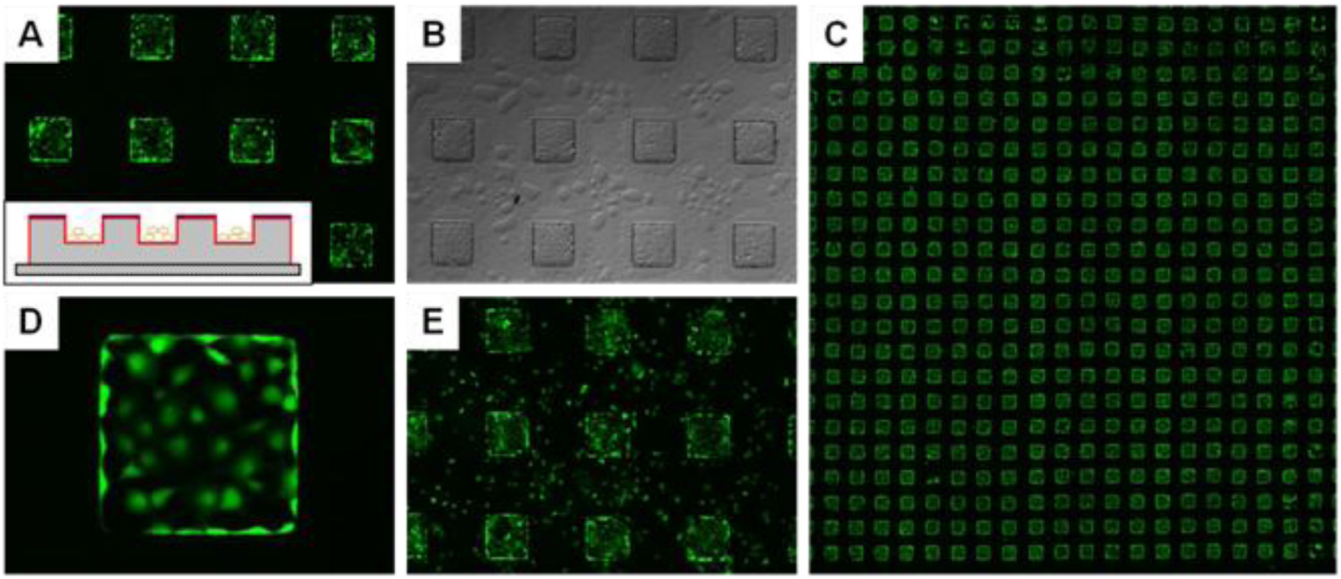


Figure 2.

Representative phase contrast and fluorescence micrographs (4X) of COS-7 cells on film-coated microwell arrays. A–D) Arrays functionalized selectively to introduce glucamine in areas between the microwells prior to cell seeding result in preferential attachment and growth of cells inside the microwells, as shown in florescence (A) and phase contrast (B) images. The images in C and D show a montage created from several different individual images showing a larger area of the array (C) and a higher magnification (20X) image showing a single well (D). The image in E shows results using an array that was not treated with glucamine prior to seeding. All images were acquired 24 h after seeding; cells were stained with calcein AM prior to imaging. Microwell dimensions are 300 μm on each side.

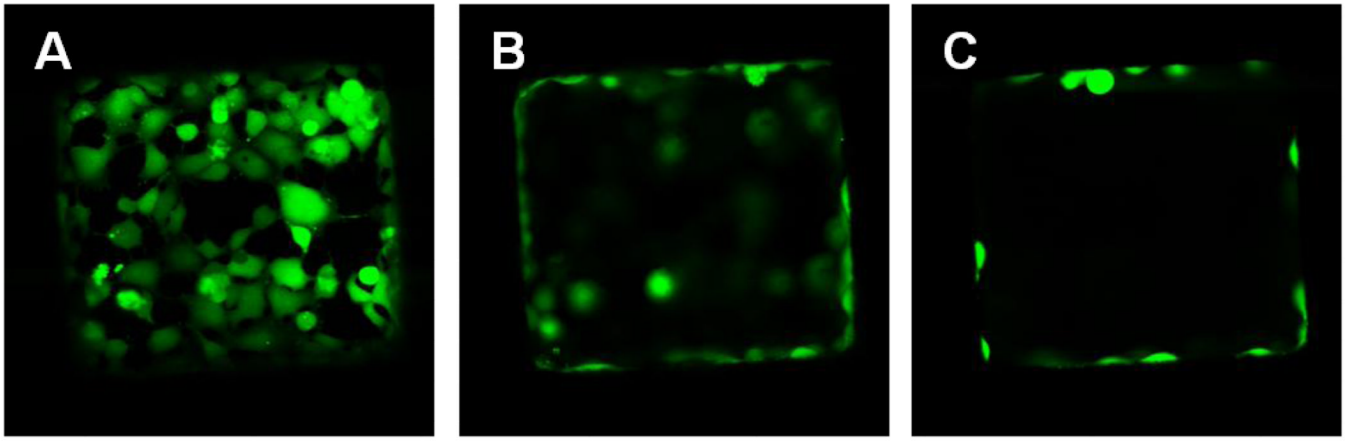


Figure 3.
Confocal fluorescent micrographs of COS-7 cells seeded on multi-layer film-coated polyurethane microwells and stained after 24 hours. Focal plane of images are at A) the bottom of the well, and B) 45 μm and C) 90 μm above the bottom.

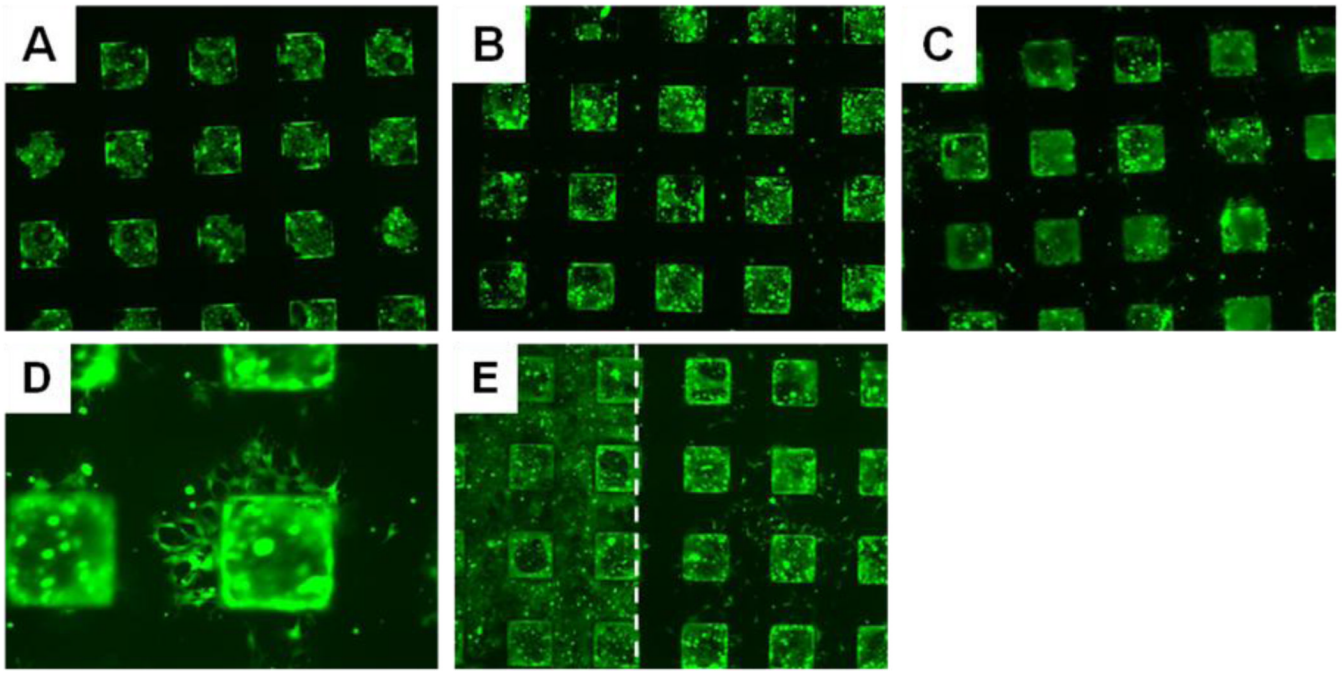


Figure 4.

Representative fluorescence micrographs (4X) of film-coated arrays seeded with COS-7 cells; films were functionalized to introduce glucamine in areas between the wells prior to seeding. Images show calcein-stained cells (A) one day, (B) 14 days, and (C) 28 days after initial seeding. The image in D shows a higher magnification (10X) image of wells from a glucamine-functionalized area acquired after culture for 21 days. The image in E shows the boundary between glucamine-reacted and non-functionalized regions of the same array, also acquired after 21 days. The dashed white line indicates the edge of the functionalized area and is included as a guide to the eye. Microwell dimensions are 300 μm on each side.

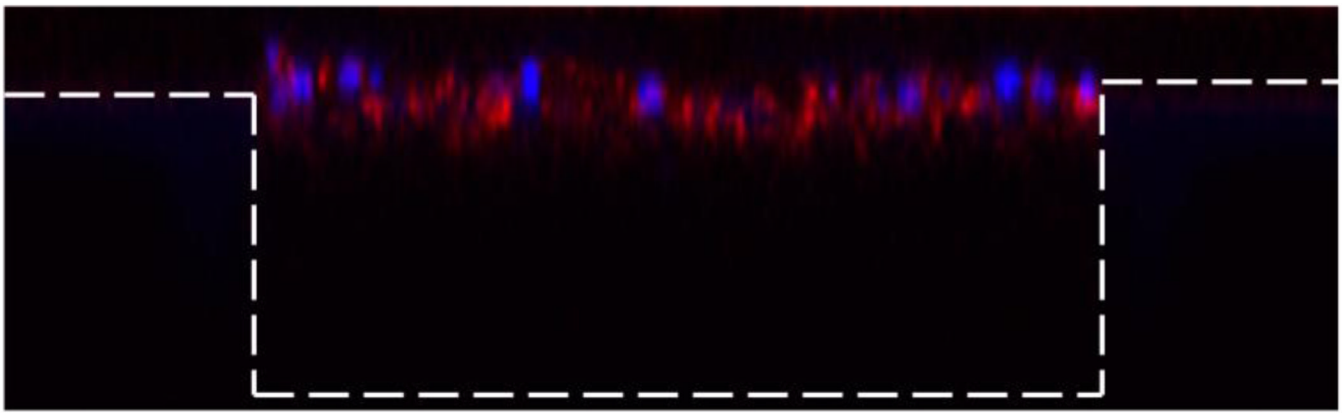


Figure 5.
Representative cross-sectional LSCM image of a film-coated microwell of a film-coated array after culturing HEK 293T/17 cells in the wells for three weeks. Cells in the topmost layer of the 3-D cell cluster were stained with Hoechst nuclear stain and WGA-594 membrane stain prior to imaging. The dotted white line indicates the surface of the microwell, which is 300 μm across and 120 μm deep, and is included to guide the eye.

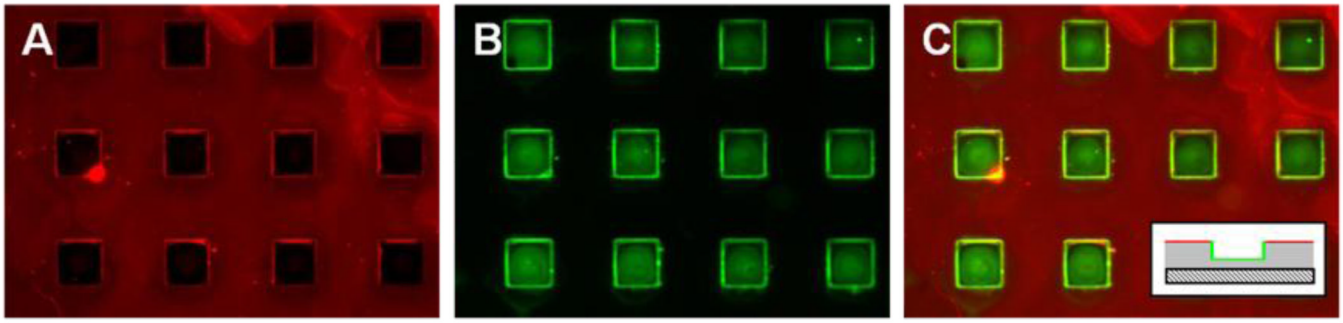
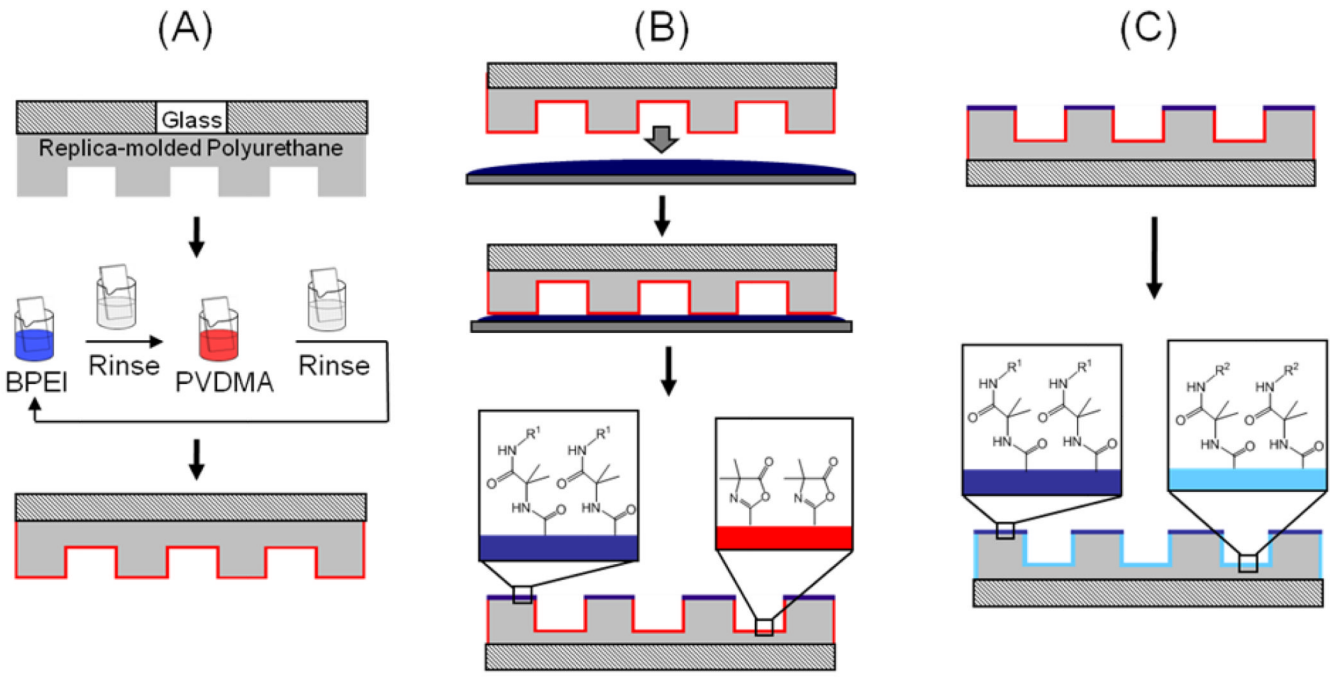


Figure 6.

Representative fluorescence micrographs (4X) of a film-coated microwell array dual functionalized using two different samples of fluorescently labeled BPEI. The array was functionalized with TMR-BPEI (shown as red) outside of the wells (using methods shown in Scheme 1B) followed by treatment with FITC-BPEI (shown as green) to functionalize the insides of the microwells (as in Scheme 1C). The images in A–C show images acquired through the red channel (A), the green channel (B), and a merged image of these two images (C). Microwell dimensions are 300 μm on each side.



Scheme 1.

Schematic illustrations of methods used to fabricate and functionalize reactive polymer multilayers on the surfaces of microwell substrates. A) Replica-molded polyurethane microwells (gray) on a glass slide (cross-hatched) were coated layer-by-layer with BPEI/PVDMA films. B) Regions on the top surfaces of microwells (i.e., areas between wells) were selectively functionalized by inverting a substrate and exposing it to a thin film of an aqueous solution containing an amine-functionalized molecule. C) Subsequent functionalization of unreacted azlactone groups inside wells was achieved by immersion of film-coated arrays in a second aqueous solution containing a different amine-functionalized molecule to yield dual-functionalized arrays.