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Fabrication of Interdigitated Micropatterns of Self-Assembled Polymer Nanofilms Containing Cell-adhesive Materials

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Abstract

Micropatterns of different biomaterials with micro- and nanoscale features and defined spatial arrangement on a single substrate are useful tools for studying cellular-level interactions, and recent reports have highlighted the strong influence of scaffold compliance in determining cell behavior. In this paper, a simple yet versatile and precise patterning technique for the fabrication of interdigitated micropatterns of nanocomposite multilayer coatings on a single substrate is demonstrated through a combination of lithography and layer-by-layer (LbL) assembly processes, termed as Polymer Surface Micromachining (PSM). The first nanofilm pattern is constructed using lithography, followed by LbL multilayer assembly and lift-off, and the process is repeated with optical alignment to obtain interdigitated patterns on the same substrate. Thus, the method is analogous to surface micromachining, except that the deposition materials are polymers and biological materials that are used to produce multilayer nanocomposite structures. A key feature of the multilayers is the capability to tune properties such as stiffness by appropriate selection of materials, deposition conditions, and post-deposition treatments. Two- and four-component systems on glass coverslips are presented to demonstrate the versatility of the approach to construct precisely-defined, homogeneous nanofilm patterns. In addition, an example of a complex system used as a testbed for *in vitro* cell adhesion and growth is provided: micropatterns of poly(sodium 4-styrenesulfonate)/poly-L-lysine hydrobromide (PSS/PLL) and secreted phospholipase A₂/poly(ethyleneimine) (PEI/sPLA₂) multilayers. The interdigitated square nanofilm array patterns were obtained on a single coverslip with poly(diallyldimethyl ammonium chloride) (PDDA) as a cell-repellent background. Cell culture experiments show that cortical neurons respond and bind specifically to the sPLA₂ micropatterns in competition with PLL micropatterns. The fabrication and the initial biological results on the nanofilm micropatterns support the usefulness of the technique for use in studies aimed at elucidating important biological structure-function relationships, but the applicability of the fabrication method is much broader and may impact electronics, photonics, and chemical microsystems.

INTRODUCTION

Discoveries in the areas of cell-biomaterial and cell-cell interactions are highly significant¹ due to the great insight they can provide into fundamental cell biology² and their potential value for medicine and biotechnology. It is generally understood that the ability to engineer and control cellular behavior is highly dependent on presentation of physical and chemical cues.¹⁻³ Therefore, development of complex functional biointerfaces, where the positioning

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of and interaction between cells of different types can be precisely controlled, requires placement of biomaterials with differing functionality in specific configurations on the same substrate.²

The capability to capture and maintain a permissive environment for multiple cell types is particularly important for cases where co-culture is desired, such as patterned neuronal networks, where incorporation of neuron-supporting glial cells are required,⁴ and hepatocyte cultures, where the presence of fibroblasts is critical for preserving characteristics of native liver tissue.⁵ While randomly-oriented co-cultures of multiple cell types have been used to better mimic *in vivo* systems, the type and degree of cell-cell interactions in such systems are not typically controllable at a desirable level. Therefore, recent efforts have targeted development of approaches to obtain cell cultures employing adhesive patterns to enhance microenvironmental control through spatial localization of multiple cell types relative to each other.^{4,6,7}

In addition to engineering systems that achieve patterning of cells on surfaces, controlling the compliance of the underlying substrate is also important. While mechanical forces have long been known to play a critical role in cellular interactions with the extracellular matrix, particularly for adherent cells,⁸⁻¹² an appreciation of substrate stiffness as a significant factor in modulating cell behavior has only recently developed.¹³ For example, recent work has demonstrated that myocytes sense differences between surfaces of varied elasticity, and express native phenotype only when exposed to substrates of stiffness typical of normal muscle.¹⁴ These findings support the concept that mechanical factors influence different cell types in fundamentally different ways, and can trigger specific changes similar to those stimulated by soluble ligands.¹⁵

The above points may be summarized by stating two key general requirements for *in vitro* systems aimed at providing scaffolding for multiple cell types: (1) the patterns must possess the appropriate physical-chemical properties to support the cells of interest and (2) the patterns must be oriented properly relative to one another.⁶ The former factor involves adhesive moieties that will selectively capture cells, non-toxicity, stability, and appropriate stiffness for the cells of interest. The second point requires a strategy for constructing the patterns that has desirable features with regard to materials that may be deposited and the relative alignment of multiple patterns.

The most commonly used biomaterial patterning techniques are based on soft lithography (microcontact printing (μ CP), patterning using microfluidic networks (μ FN), elastomeric membranes, and laminar flow patterning),¹⁶⁻²⁰ and employ elastomeric micro-molds made of poly(dimethylsiloxane) (PDMS), as well as conventional photolithography.²¹ The soft lithography techniques have advantages that include rapid prototyping, low cost, and the ability to pattern on non-planar substrates. Of these, microstamping is one that has found widespread use in biological studies; however, alignment of separate elastomeric stamps across large areas is difficult to achieve, and therefore deposition of patterns of different types with desired relative alignment is not practiced.

A handful of examples of multicomponent patterning of adjacent biomaterial patterns on a single substrate have been reported, including multilevel stamps,²² 2-D & 3-D microfluidic networks,²³⁻²⁵ photolithography,²¹ thermo-biolithography,²⁶ and photochemical reactions²⁷⁻³⁰ combined with lithography. These approaches may potentially be adapted to pattern multilayer films; however, each has its own advantages and disadvantages. For example, due to the elastomeric nature of PDMS, large-area patterning with 2-D or 3-D microfluidics is problematic. Thermo-biolithography suffers from low resolution and difficulty in alignment of multiple stamps during pattern transfer.

In addition to the disadvantages noted, none of the techniques described above can provide significant control over the elasticity of the materials deposited. In contrast, the ionic layer-by-layer (LbL) self-assembly technique,^{31,32} an elegant method with ability to deposit multilayer films of highly interpenetrated, precise, and uniform nature with minimal constraints on the size, geometry, or materials,³³ can be applied to obtain multilayer structures with varying composition, thickness (from few nanometers to microns), roughness, elasticity, porosity, etc. The compliance of multilayer nanofilms is highly tunable, as the effective modulus depends not only on the constituent molecules, but also on the assembly conditions and the number of adsorbed layers. Multilayer films have been shown to exhibit thickness-dependent stiffness when deposited on solid substrates, and the effective modulus was found to increase tenfold after crosslinking (e.g. ~50 kPa up to ~800 kPa for native and crosslinked poly(L-lysine)/hyaluronan multilayers, respectively).³⁴ Much higher moduli have been reported for PAH/PSS nanofilms, ranging from 100s of MPa in the hydrated state to GPa when dried.³⁵ These nanofilms can be constructed from functionalized macromolecules, enzymes, and proteins embedded at different depths to obtain complex biomimetic nanoarchitectures,³⁶⁻⁴² and a number of recent studies have described the use multilayers of biomaterials for cell-related applications.⁴³⁻⁴⁶ However, several of these studies share a common limitation in that the multilayer films are homogeneous across the plane. Thus, while LbL can enable tuning of substrate physical-chemical properties, including stiffness, a suitable micropatterning technique is required for the spatial definition of multicomponent micropatterns of these multilayers.

Two-component multilayer films have been patterned using a technique based on microcontact printing. Chemically patterned surfaces were utilized to construct polymer-on-polymer⁴⁷ structures and side-by-side⁴⁸ structures of polymers & colloidal⁴⁹ particles, and patterns of materials adhesive to one cell type amidst a background of material adhesive to another cell type have been demonstrated.^{6,7} These processes require a balance between the use of interactions that could prevent deposition (steric and hydration forces or electrostatic repulsion) and those that could encourage it; therefore, the materials that can be employed are limited. Furthermore, micropatterning of more than two components is a challenging task because the technique is based on the presence of two different, complementary functional groups on the substrate.

Multilayer transfer printing (MTP), another soft lithography based technique, is a recent development for fabricating multicomponent multilayer patterns.⁵⁰ In this method, polyelectrolyte multilayers formed on the surface of a PDMS stamp were directly transferred onto a surface with a charge opposite to that of the top most layer of the multilayer film printed. Despite the promise this brings for versatile micropatterning of nanocomposite films, two significant issues appear to limit the approach: First, as with all stamping approaches, a precise mechanical positioning system is required to obtain aligned multicomponent patterns. This limitation has recently been resolved by implementing an alignment system in the MTP process, though the misalignment is directly proportional to the size of the stamp used, with a misalignment of 50 μm (18 % deviation) for a $2.8 \times 3.3 \text{ cm}^2$ stamp.⁵¹ Second, the MTP process needs a fine balance of forces between the bottom-most layer of the multilayer films and the PDMS stamp (that would become the top-most layer after the multilayer films are transfer printed), which must be optimized for different materials.

In summary, with the goal of constructing cell-adhesive patterns with precisely-tailored physicochemical properties, including stiffness, existing techniques present severe limitations. Use of multilayer nanofilms for creating appropriate scaffolds is desirable, and a straightforward technique to construct multilayer nanofilm scaffolds with few restrictions on materials and excellent alignment of subsequent patterns is needed. It appears that combining photolithography with LbL assembly can overcome many of the shortcomings of current

methods for multilayer film patterning.⁵² The LbL-liftoff (LbL-LO) technique,⁵³ which combined the lithography and LbL techniques with a single lift-off step for the patterning of such surfaces, was previously demonstrated for the fabrication of two-component “comparison chips” with one component (protein/polypeptide) on each half of a single substrate.⁵⁴ However, this limited capability must be further extended to a multi-step procedure enabling deposition of adjacent, overlapping, and/or interdigitated patterns.

Therefore, this report describes a simple yet versatile and precise process which provides the capability to engineer complex 3D multilayers in aligned interdigitated micropatterns on a single substrate, such as that illustrated in Figure 1. The technique, termed Polymer Surface Micromachining (PSM), involves a combination of lithography and LbL methods and has the primary advantage of well-controlled alignment for registration of subsequent patterns of multilayer films. Therefore, complex functional biointerfaces providing different chemical and physical cues may be easily constructed, offering the realization of a wide range of systems requiring precise arrangements of physicochemical features, and could be used for a wide range of related biological applications, including biological testbeds for basic biological studies, multicellular communication & organizational studies, biosensors, drug screening, and tissue engineering. The basic approach may also be applied to constructing complex patterns of self-assembled materials into, for example, polymeric electronic devices, optical systems, or other systems in which nanocomposite materials are required, and the approach can potentially be combined with more standard surface micromachining techniques to further extend the range of applications.

EXPERIMENTAL

Materials

Microscope cover glasses (24×60mm) used as substrates for the nanofilm patterning were purchased from VWR International. Nano-Strip™ was purchased from CYANTEK Corporation. Poly(diallyldimethylammonium chloride) (PDDA) (Mw ~100–200kDa), poly(sodium 4-styrenesulfonate) (PSS) (Mw ~1MDa), poly(ethyleneimine) (PEI) (Mw ~750kDa), poly-L-lysine hydrobromide (PLL) (Mw ~25,700Da), poly(allylamine hydrochloride) (PAH) (Mw ~70KDa), and fluorescein isothiocyanate (FITC) were purchased from Sigma-Aldrich. Secreted phospholipase A₂ (sPLA₂, Type III, from bee venom) (Mw ~14kDa) was purchased from Cayman Chemical. Tetramethylrhodamine-5-(and-6)-isothiocyanate (TRITC) and Texas Red-X, succinimidyl ester (MW ~816.94Da) were ordered from Molecular Probes. Cy™5-bis-NHS ester was ordered from Amersham Biosciences. Positive photoresist, S1813, and positive resist developer, MF-319, were obtained from Shipley. All chemicals of commercial origin were used as received.

Preparation of Polyelectrolyte and Polypeptide Solutions

Solutions of PDDA, PAH, and PSS with concentration of 2 mg mL⁻¹ with 0.5 M KCl and a solution of 2 mg mL⁻¹ PEI were prepared in DI H₂O for use in self-assembly. PLL, PAH, and PEI were labeled with TRITC, (Texas Red and Cy5), and FITC, respectively, using standard procedures.⁵⁵ PLL, PAH, and PEI were separated from dye solution by precipitation with acetone, centrifugation, and resuspension in aqueous solution. Solutions for deposition were prepared as 167 µg mL⁻¹ in DI water for TRITC-PLL and 400 µg mL⁻¹ in 0.1M sodium bicarbonate at pH 9.0 for sPLA₂.

Lithography

Substrates were incubated in Nano-Strip™ at 70 °C for 1 hr to remove organic contaminants and create a uniform negative charge on the substrates, then they were rinsed in DI water and dried using N₂. A precursor layer of PDDA was then deposited on the negatively-charged

substrates by incubating in PDDA for 20 min, rinsing in DI water, and finally drying in N₂. The choice of PDDA as a precursor layer was based on previous observations, where PDDA had proven to be a cell-repellent material for smooth muscle cells,^{53,56} and preliminary screening studies, where similar results were observed when tested with neuronal cells.⁵⁴ However, in principle any other cytophobic material, providing the required charge, could replace PDDA or, alternatively, the background surface could be backfilled with a neutral cytophobic material (e.g. PEG) after the completion of all the patterning steps. Photoresist S1813 was spun on the substrates (1000 rpm - 100 r s⁻¹ - 10 sec, 3000 rpm - 500 r s⁻¹ - 50 sec), soft baked at 115 °C for 1 min, and photo-patterned using UV radiation (365/405 nm, 7 mW cm⁻²) for 15 sec. Patterns were developed for 20 sec, then substrates were rinsed in DI water and dried using N₂.

Layer-by-Layer Assembly

For all the LbL assembly processes, a basement multilayer of {PSS/PDDA}₃ was deposited on the patterned substrates through immersion in the PSS or PDDA solutions for 10 min, rinsing in DI water, and finally drying in N₂. This precursor multilayer deposition of strong polyelectrolytes is an important step to attain a uniformly charged surface for subsequently deposited layers. The process of rinsing in DI water and drying with N₂ was repeated after each deposition step throughout the LbL assembly process, since it was found to help to obtain undistorted multilayer patterns in the final development stage.

After the first round of photoresist development and the LbL assembly of precursor layers, a nanofilm architecture of {sPLA₂/(FITC-PEI)}₄/sPLA₂ was deposited on top of the patterned substrates with pre-existing {PSS/PDDA}₃ films. This was accomplished through deposition of four bilayers of sPLA₂/(FITC-PEI) by alternate exposure of substrates to negatively-charged sPLA₂ and positively-charged FITC-PEI solutions, followed by a fifth and final layer of sPLA₂. The optimum adsorption times (minimum time required for the resaturation of polyion adsorption that results in the charge reversal) were previously determined through QCM experiments to be 20 min for TRITC-PLL and 50 min for sPLA₂.⁵⁴ Hence, the substrates were immersed in sPLA₂ and FITC-PEI for 50 min and 10 min, respectively. The lift-off process was performed by sonicating the substrates in acetone for 3–5 min to obtain the first set of patterns.

Multi-Component Scaffolds

A second lithography step was performed on the substrates, this time with a set of patterns aligned with the first set of patterns using previously patterned alignment marks, and a multilayer architecture of {PSS/(TRITC-PLL)}₅ was deposited in a procedure similar to that used for {sPLA₂/(FITC-PEI)}₄/sPLA₂. Here, PSS was used as the polyanion, and adsorption times were 10 and 20 min for PSS and TRITC-PLL, respectively. For the four-component systems, a similar procedure was followed. The adsorption time was 10 min for PAH-Cy5 and PAH-Texas Red.

Characterization

Fluorescence microscopy was used for imaging the resulting multicomponent micropatterns to demonstrate successful multicomponent patterning and to assess the uniformity and spatial registration using an epifluorescence microscope (Nikon, Model-Eclipse TS100) equipped with a Nikon COOLPIX995 digital Camera. The exposure time used with the 40X objective was 2 s for the FITC cube and 0.25 s for the TRITC cube. Digital zoom settings of F3.0 were used for the imaging process. Images were taken sequentially using a FITC cube followed by a TRITC cube. The FITC cube contained a long-pass emission filter. Confocal laser scanning microscopy (CLSM) (Leica, Model-TCS SP2) was also used to perform sequential FITC-

TRITC imaging and Cy5-FITC-TRITC imaging of the two- and four-component substrates, respectively, at optical magnifications of 10X and 63X.

Surface profilometry was used for assessment of the topographical differences of the multicomponent micropatterns. The surface profiler (KLA-Tencor, Model-Alpha-Step IQ) was used to collect line scan structural data of the patterns on three different substrates. The vertical dimensions of the patterns were obtained directly from the line scan measurements. Final height values were based on an average of three measurements. The scan parameters used were as follows: stylus force of 20 mg, scan length of 200 μm , scan speed of 20 $\mu\text{m s}^{-1}$, and sampling rate of 50 Hz. Stylus profilometer height measurements were confirmed with tapping-mode AFM and optical measurements.

A spectroscopic ellipsometer (SENTECH, Model-SE 850) was also used to measure the thickness of PSS/PDDA multilayers. The measurements were performed at an incidence angle of 70° and the 250–850 nm spectral range was used. A refractive index of $n_0=1.5$ was assumed for thickness calculations using the SPECTRARAY software.

Cell Culture

As a specific example of how this technology may be applied to biological studies, primary cultures of cortical neurons were used as a model biological system to compare the results with those obtained using side-by-side “comparison chips”.⁵⁴ While poly-L-lysine is well known and widely used as a neuronal cell adhesion material, we have recently observed preferential attachment of neurons to secreted phospholipase A₂ (sPLA₂), which is a low molecular weight transcellular enzyme that has been shown to be involved in digestive and inflammatory response mechanisms, and is known to have potent deleterious effects on neurons of the central nervous system.^{57,58} Since sPLA₂-binding proteins and receptors have been identified in muscle and brain cells, the enzymatic and signaling functions of sPLA₂s are believed to have cell surface targets, so these observations are not stunning.⁵⁹⁻⁶¹ However, the initial experiments showing that primary cortical neurons preferentially bind to sPLA₂ when presented with both sPLA₂- and PLL-terminated nanofilm patterns were performed with the two patterns on different halves of the same chip; therefore, the question of whether spatial separation plays a key role in this attachment behavior has been raised.⁵⁴

The multicomponent patterning method described here provides the ability to spatially orient the two sets of micropatterns containing PLL and sPLA₂ in different arrangements. Therefore, to compare the results from the current technique with the results obtained through comparison chips,⁵⁴ interdigitated two-component nanofilm patterns of PLL and sPLA₂ were constructed. For these experiments, primary cultures of cortical neurons were prepared as previously described⁶² from embryonic day 15 rat embryos and grown on the patterned substrates in 37 °C, 5 % CO₂ incubators. Cortical neurons grown on the multicomponent micropatterns were imaged using a Nikon inverted microscope and a Roper Scientific CoolSnap HQ camera with Metamorph software (Universal Imaging).

RESULTS & DISCUSSION

The cartoon in Figure 2 depicts the fabrication process flow used in this work. The first step in the fabrication was the treatment of the substrates with Nano-Strip™ followed by the deposition of the PDDA precursor layer. The next step was the patterning of substrates using S1813. The patterned substrates were then modified using LbL self-assembly with the desired multilayer configuration. Lift-off was performed on the substrates to obtain the first set of multilayer patterns. This procedure was repeated to obtain the second set of multilayer patterns, and could be repeated as necessary to deposit additional nanofilm patterns on the same substrate. This method is analogous to surface micromachining, except that the deposition

materials are polymers and biological materials that are used to produce multilayer nanocomposite structures.

Two-Component Scaffolds

Figure 3 contains fluorescence and phase contrast images of the micropatterns fabricated using the PSM method. Figure 3(a) is an image of 20 μm interdigitated square patterns with an offset of 50 μm in both vertical and horizontal direction. The green patterns are multilayer heterostructures with a configuration of $\{\text{PSS/PDDA}\}_3/\{\text{sPLA}_2/(\text{FITC-PEI})\}_4/\text{sPLA}_2$, whereas the red patterns possess an architecture of $\{\text{PSS/PDDA}\}_3/\{\text{PSS}/(\text{TRITC-PLL})\}_5$. Figure 3(b) is an image of similar interdigitated patterns, with an offset of 50 μm in the vertical direction only. Figure 3(c) is a phase contrast image of nanocomposite sPLA₂ and PLL patterns. The dark squares are nanocomposite PLL patterns, whereas the bright squares are nanocomposite sPLA₂ patterns. These images prove that the current patterning technique provides the capability of registering micropatterns in the desired configuration, with high precision and accuracy. The average misregistry, M ,⁶³ between the patterns of the two components, was found to be 0.79 μm . For an offset length of 50 μm , this misregistry relates to an error of 1.58 %. This error increases for smaller lengths. However, most of the times very small lengths are not required for biological studies related to cells. Figure 3(d) is an illustration representing the cross-sectional side view of the patterns. It is noteworthy that when the total number of layers for the different components is nearly the same, the height difference is substantial.⁵⁴ The thickness of $\{\text{PSS/PDDA}\}_3$, measured using an ellipsometer, was found to be 18.2 ± 0.09 nm. The thicknesses of PSS/PLL and PEI/sPLA₂ multilayers were estimated by subtracting the thickness of three bilayers of PSS/PDDA from the total height values measured through surface profilometry as noted below.

In Figures 3(a)-(b), the fluorescence intensities of the nanocomposite patterns are uniform within a pattern, indicating that the biomaterials were deposited uniformly. In Figure 3(c), it can be seen that the micropatterns have clear, sharp, and defined edges that are faithful reproductions of the photomask. There were no obvious traces of residual photoresist or multilayer nanofilms on the PDDA background regions. Furthermore, these images indicate that using the current method, complex heterostructures of multiple components may be easily obtained with simplicity and high fidelity (edge resolution of the patterns is <1 μm) to spatially orient the nanocomposite polymer film micropatterns with precise alignment. However, it is also obvious that the corners of the squares, which are supposed to be sharp, are slightly rounded, but the purpose of this paper was to demonstrate the current technique, and not to optimize it. Better edge resolution and sharp corners can certainly be achieved by optimizing the process parameters. We have found that this is dependent on the material (molecular weight, size of molecule) being deposited photoresist thickness used, and the number of multilayers deposited, as well as the solution conditions (pH, ionic strength) affecting the multilayer formation. These factors are currently being characterized to optimize the method for more accurate transfers of photomask details, and specifics will be described in a future report.

Figure 4 contains line scan structural data of interdigitated nanocomposite square micropatterns of sPLA₂ and PLL, with multilayer configuration of $\{\text{PSS/PDDA}\}_3/\{\text{sPLA}_2/(\text{FITC-PEI})\}_4/\text{sPLA}_2$ and $\{\text{PSS/PDDA}\}_3/\{\text{PSS}/(\text{TRITC-PLL})\}_5$, respectively. The height difference, as depicted schematically in Figure 3(d), can clearly be seen from the profile; based on an average of three measurements, the nanocomposite PLL patterns were 81.53 ± 1.31 nm thick, whereas the sPLA₂ patterns were 21.33 ± 0.71 nm thick. This demonstrates the ability of the current technique to fabricate complex 3D multilayers in aligned interdigitated micropatterns. The standard deviations indicate that the heights of the nanocomposite patterns across different substrates are very uniform (~ 1.6 % and ~ 3 % variations for PLL and sPLA₂ patterns, respectively). The inherent ability of the technique to accommodate different surface

chemistries and finely tune the topographies will be useful in presenting varying physical and chemical cues to influence cell growth.^{64,65}

The difference in heights of the sPLA₂ and PLL patterns is in agreement with the previously published results from quartz crystal microbalance (QCM), surface profiler, and AFM measurements.⁵⁴ The shorter heights of sPLA₂ patterns compared to PLL patterns of similar multilayer architecture can be attributed to the molecular size and weaker net charge of sPLA₂. However, if scaffolds mimicking complex 3D *in vivo* conditions more closely are to be realized, the heights of the patterns could be easily adjusted during the multilayer LbL assembly process by depositing more or fewer layers as necessary.

Cell Culture

Initial tests on the applicability of these multicomponent heterostructures to biological studies, performed through preliminary cell culture experiments, resulted in interesting observations. Figure 5 contains the TRITC, phase, FITC, and merged TRITC+FITC+phase images of 3D interdigitated multicomponent nanocomposite micropatterns of PLL and sPLA₂, with multilayer configuration of {PSS/PDDA}₃{PSS/(TRITC-PLL)}₅ (red) and {PSS/PDDA}₃{sPLA₂/(FITC-PEI)}₄/sPLA₂ (green), respectively, acquired at an original microscope magnification of 400X. The patterns are 20 × 20 μm squares separated by 80 μm, connected by 5 μm wide lines, with the two material patterns offset by 50 μm and 40 μm in the vertical and horizontal directions, respectively. The area shown is at the end of a line of patterns; thus the FITC patterns extend 50 μm beyond the TRITC patterns. The “halo” appearing adjacent to the fluorescent patterns in the merged image is due to the imaging procedure, and is not a result of diffusion of the fluorescent molecules away from the patterns. According to observations, the patterns are stable in aqueous solution and cell culture media for at least 5 weeks, with no apparent loss of fluorescence intensity, which agrees with other reports on the stability of multilayer nanofilms.^{33,66,67}

For observations performed up to 2 weeks *in vitro*, it was found that neurons preferentially bound to {PSS/PDDA}₃{sPLA₂/(FITC-PEI)}₄/sPLA₂ patterns compared with {PSS/PDDA}₃{PSS/(TRITC-PLL)}₅ patterns. In addition, large and fine neuronal processes were observed, following preferential localization of cells on the FITC patterns. There is no binding of neurons to the nanocomposite PLL patterns, although the large processes do not appear to see the 80 nm tall PLL micropatterns as an obstacle from interacting with neurons binding on other sPLA₂ micropatterns. Also, from these images it is obvious that PDDA proves to act well as a cytophobic material. It is noteworthy that for the data presented here, the sPLA₂ patterns were deposited first, followed by the deposition of the PLL patterns; identical behavior was observed in experiments where the order of deposition was reversed.

In earlier studies, it was shown that when neurons were cultured on substrates with only PLL patterns, they attached to the patterns and were confined to the patterns;⁶⁸ however, our results suggest that the neurons preferred sPLA₂ patterns over the PLL patterns. These results agree with and support the cell adhesion behavior observed with the half sPLA₂/half PLL comparison chips,⁵⁴ but now any possibility of preferential attachment due to position on the surface has been removed, since squares composed of different biomaterials are adjacent to each other and interdigitated. Thus, the primary neurons used for these investigations appear to select sPLA₂ over PLL as the substrate of choice.

The exact reason behind the observed result of preferential binding of neuron cells to sPLA₂ is not obvious, though several possibilities exist. One explanation could be that sPLA₂ composite nanofilm patterns are thinner in the z-axis than the PLL patterns (~20 vs. ~80 nm), as shown schematically in Figure 3(d) and Figure 4. Additional investigation of the contribution of surface topographical cues towards the specific binding of neuron cells to sPLA₂ is currently

underway, and preliminary findings suggest there is indeed a dependence of the binding behavior on the nanoscale film thickness.⁶⁹ Whether the observations are truly height-dependent, or are also influenced by substrate stiffness of the substrate,¹³ which also varies with film height,^{34,35} is another question to be answered in future work. Regardless of the true nature of the interactions and details of the factors involved, the cell culture results indicate that the current technique could find immediate application in constructing precise co-culture systems by specifically capturing multiple cell types with defined spatial arrangement for better understanding of cell-cell interactions.^{4,5,70-72}

From these observations, it can be deduced that this patterning technique may be successfully applied to the fabrication of 3D multilayer multicomponent micropatterns and may aid in studying biological or biochemical pathways. It is noteworthy that the use of acetone might be expected to cause substantial degradation of biological function for patterned molecules. However, previous observations and the current results suggest that the features of the molecules that are responsible for cell binding (e.g. those with specific interaction with cell membrane molecules) are retained through the process, at least for sPLA₂ and PLL used here and also for fibronectin and gelatin.⁵⁶ Furthermore, nanofilms of glucose oxidase, for which activity assays are sensitive and straightforward, did not exhibit significant loss of activity when exposed to acetone. Thus, the nanofilm supports appear to provide a degree of anchoring and conformational stability that is sufficient to protect these molecules from major damage, though it is not immediately clear how general this is and what classes or sizes of molecules will exhibit similar behavior. It is also worthwhile to note that, if there are adhesion ligands or other molecules of interest that do prove sensitive to the solvent exposure, the PSM method described here could be modified to work with these more delicate materials by implementing biocompatible photoresists and developers in the lithography processes.⁷³⁻⁷⁶

Four-Component Scaffolds

In order to demonstrate the ability of the PSM method to fabricate more complex systems, patterns of four different components were fabricated on the same substrate. Micropatterns of the four different components had a multilayer configuration of {PSS/PDDA}₃{PSS/(Cy5-PAH)}₅ (blue squares), {PSS/PDDA}₃{PSS/(TRITC-PLL)}₅ (light red squares), {PSS/PDDA}₃{PSS/(FITC-PEI)}₅ (green squares), and {PSS/PDDA}₃{PSS/(Texas Red-PAH)}₅ (bright red squares). Figure 6 contains the sequential Cy5-FITC-TRITC (TRITC=wide emission bandwidth, allowing simultaneous Texas Red and TRITC imaging) scanning confocal images obtained using a CLSM. Figure 6(a) contains the individual sequential scanning images for Cy5, FITC, and TRITC, along with the overlay image, imaged at an optical magnification of 10X. Figure 6(b) contains the overlay image of similarly imaged sequential scanning images at an optical magnification of 63X. Figure 6(c) contains the overlay image of digitally zoomed sequential scanning images. Finally, Figure 6(d) is a graph of average intensities for lines across the square patterns, which gives an indication of the homogeneity of the fluorescence.

These images clearly illustrate the aligned micropatterns of four different components. The fluorescence intensities of the nanocomposite patterns are uniform within a pattern as well as over a large area on the substrate, indicating that the biomaterials were deposited uniformly. The fluorescence homogeneity was quantified by calculating average and standard deviation of intensity for 50 × 50 pixel square regions within the square patterns before image processing, and was found to be ~5 % for the brighter Cy5 and Texas Red patterns and ~11 % for the TRITC and FITC patterns. From Figure 6(a), it can be seen that the four components are well aligned over a large area, and there is no obvious cross-contamination of fluorescence, indicating that even though the current technique involves repetition of lithography, LbL assembly, and lift-off processes, the different biomaterials are confined to the patterned regions

in which they are deposited. Overall, these images prove the ability of the PSM method to fabricate aligned multilayer multicomponent patterns.

CONCLUSIONS

A simple yet versatile and precise patterning technique to fabricate 3D multilayer multicomponent heterostructures of bioactive coatings on a single substrate has been demonstrated. This method is analogous to surface micromachining, except that the deposition materials are polymers and biological materials that are used to produce multilayer nanocomposite structures. The fabrication results, metrology results, and the initial results from the biological studies on the multicomponent micropatterns prove the success and usefulness of the method. The ability to obtain multicomponent heterostructures with great precision and simplicity overcomes some of the constraints of existing techniques, and the process can be easily integrated into existing automated systems used for lithography and LbL assembly. The metrology results indicate that nanocomposite micropatterns with varying physical and chemical cues could be easily integrated into scaffolds using the current technique.

The cell-culture results indicate that the lithographic processes undertaken during this technique have minimal or no deleterious effects on the biological model being tested. The metrology and cell-culture results for the two-component nanocomposite micropatterns and the fabrication results for the four-component nanocomposite micropatterns together prove the importance of the immediate applicability of the current technique towards studying cell-biomaterial interactions in a whole new fashion. The current technique could play an important role in stem cell research, as a tool to understand biochemical pathways of proliferation, migration, and differentiation under different physicochemical conditions, since stem cells demonstrate plasticity dependent on their environment.⁷⁷⁻⁸¹ Also, defined spatial arrangements of anchorage-dependent cells could create a high level of complexity in co-cultures and could be used as a tool for analyzing stem cell behavior under various biophysicochemical conditions.^{6,7,82} Time-varying signals could also be easily presented to the cells by integrating biodegradable materials into the multilayer assembly process,⁸³⁻⁸⁶ thereby dynamically influencing the cell behavior. Although the intended purpose for developing 3D multilayer multicomponent micropatterns is to produce novel bioactive systems, their applicability is more general and may find use in a broad range of applications including electronics, photonics, optoelectronics, and chemical and biochemical sensors.

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Figure 1.
Schematic illustration of multiple 3D multilayer micropatterns.⁵⁴

Figure 2.

Template used for PSM method (a), (b) Photolithography of pretreated substrate, (c) LbL deposition of component 1, (d) After first Lift-off, (e) Second photolithography step, (f) LbL deposition of component 2, (g) After second Lift-off, (h) Top view of the chip.

Figure 3.

3D interdigitated multicomponent micropatterns constructed on glass substrates: Images of 20 μm patterns (40X) collected through FITC cube with the second component (a) Offset by 50 μm in vertical and horizontal direction, (b) Offset of 50 μm in vertical direction [Green: {PSS/PDDA}₃/sPLA₂/(FITC-PEI)₄/sPLA₂ and Red: {PSS/PDDA}₃/PSS/(TRITC-PLL)₅], (c) Phase contrast image [Dark squares: PLL patterns and Bright squares : sPLA₂ patterns], (d) Cartoon of the cross-section view of patterns.

Figure 4. Surface profiler line scan structural data of 3D interdigitated multicomponent nanocomposite micropatterns of PLL and sPLA₂.

Figure 5.

Tiled TRITC fluorescence, phase, FITC fluorescence, and merged phase+TRITC+FITC images (Top to bottom) of primary rat cortical neurons grown on $\{\text{PSS/PDDA}\}_3/\{\text{sPLA}_2/(\text{FITC-PEI})_4/\text{sPLA}_2\}$ patterns (green) next to $\{\text{PSS/PDDA}\}_3/\{\text{PSS}/(\text{TRITC-PLL})_5\}$ patterns (red). Neurons were plated onto nanofilm substrates at a density of 100,000 cells/ml in 2.7 ml of culture medium in a 35 mm Petri dish. Images were captured after 2 weeks *in vitro*. As is obvious from the location of cells on the surfaces, preferential attachment of neurons to sPLA₂-terminated films was observed.

Figure 6.

(a) Sequential Cy5-FITC-TRITC scanning CLSM images and overlay image at 10X, (b) Overlay image at 63X, (c) Digitally zoomed overlay image showing one pattern each for the four-component patterns: Blue- $\{PSS/PDDA\}_3/\{PSS/(Cy5-PAH)\}_5$, Light red- $\{PSS/PDDA\}_3/\{PSS/(TRITC-PLL)\}_5$, Green- $\{PSS/PDDA\}_3/\{PSS/(FITC-PEI)\}_5$, and Bright red: $\{PSS/PDDA\}_3/\{PSS/(Texas Red-PAH)\}_5$; (d) Linescan intensity data for image in (c), before processing: Blue long dash-Cy5, Red-TRITC, Maroon dash-dot-Texas Red, Green short dash-FITC.