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Novel High-Resolution Micropatterning for Neuron Culture Using Polylysine Adsorption on a Cell Repellant, Plasma-Polymerized Background

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Abstract

The ability to organize individual neurons and their processes in culture provides important benefits to both basic neuroscience research applications and the development of biomedical microdevices. While numerous methods have been used to produce such micropatterning of neurons and cells in general, there has yet been no method to simultaneously provide high-resolution patterns with high compliance of cells to desired patterns and good manufacturability. To develop such a process, this work used a plasma polymerized, nonfouling poly ethylene oxide (PEO)-like film to provide a cell repellant substrate on which cell adhesive micropatterns can be selectively laid down. While the use of plasma polymerized, organic films have been used for cell micropatterning, this process exploits the often-overlooked tendency for the surface of this PEO-like material to adsorb polylysine from aqueous solution while remaining nonfouling with respect to other species, such as bovine serum albumin (BSA) and immunoglobulin G (IgG). When the adsorption of polylysine was enhanced by brief plasma oxidation, which slightly alters the surface chemistry of the material, simple photolithographic liftoff could be used to micropattern stable, cell adhesive areas on an otherwise cell repellant background. We showed that the application of photolithography itself on the PEO-like material did not alter its chemical properties, nor did it result in the erosion of the micropatterned polylysine on its surface. Hippocampal neurons from embryonic mice flourished on these micropatterned substrates and exhibited viability comparable to neurons cultured on polylysine coated glass. Furthermore, the compliance of cell bodies and outgrowing neurites to the micropatterns was nearly perfect. In addition to providing cell adhesive regions, the micropatterned polylysine coating also served as a template mediating the immobilization of other bioactive species such as IgG and laminin. Using this “piggybacking” of laminin on polylysine, we were also able to culture and micropattern retinal ganglion cells (RGC).

1. Introduction

Neurons are highly polarized cells with distinct cell bodies and long, slender processes known as axons and dendrites. For culturing of neurons in vitro, the use of microscale patterns of cell adhesive material deposited on culture substrates has enabled researchers to precisely control the positioning and orientation of individual neurons. There is widespread interest in organizing or patterning neurons and their axons and dendrites along precise geometries to form simple neural circuits that can be aligned with structures like microelectrode arrays.^{1–5} Microscale patterning of neurons has also been useful in applications ranging from biosensors and neuro-electronic interfaces to neuronal culture platforms serving basic research needs.^{4,6–8} Naturally,

optimal neuron patterning techniques that can address these needs must produce high compliance of the cultured cells to the desired geometries and include patterns with high spatial resolutions to organize not only the neuronal cell bodies but also the neurites (axons and dendrites), which are only a few microns wide. Additionally, such patterning techniques should be produced using a reliable fabrication process that allows high-volume manufacture of the patterned substrates, and result in biologically active patterns that can be stored for extended periods of time.

Because of the widespread applications of cell patterning, numerous methods have been developed over the years to deposit and pattern cell adhesive material at the microscale, including the patterning of adhesion proteins using photolithographic liftoff⁹ or a variety of “soft lithographic” techniques^{1,2,8,10} such as the popular microcontact printing (μ CP),^{1,11} and even direct patterning by laser ablation of molecular monolayers.^{2,12} To attain more effective cell patterning, a nonfouling, cell repellant material is sometimes established alongside the cell adhesive micropatterns to further enforce the compliance of cells and their processes to the desired patterns.^{1,13} Nevertheless, while many existing methods can produce patterns suitable for neurons, a simple, reliable technique that can simultaneously provide molecular patterns with high spatial resolution, manufacturability, and long shelf life remains elusive.

The low temperature deposition of robust, thin organic films via plasma-induced polymerization of monomeric precursors, considered a form of plasma-enhanced chemical vapor deposition (PE-CVD), has recently provided a new format for creating patterned cell culture.^{14–20} A key material developed for this application is a nonfouling, cell-repellant polyethylene oxide (PEO)-like material, plasma polymerized from vapors of diglycol methyl ether (or any of several similar species) and deposited to fully blanket any cell culture substrate.^{16,21} Early applications of this material used photolithographic lift-off to directly pattern the deposition of the PEO-like material.^{22,23} However, the PEO-like material has also been used as a blanket cell repellant foundation on which bioactive species were introduced via μ CP^{22–24} or on which other types of organic films—varieties that promote cell attachment—were patterned.^{14,17} Subsequent work introduced the concept of “tuning” or selectively altering the surface properties of the PEO-like film itself to render it cell adhesive only on the desired areas. For example, applications of microwave-generated Ar/H₂ plasma¹⁵ or electron beam lithography²⁵ have been used to tune the PEO-like character and the surface topography to render specific regions cell adhesive, while leaving adjacent areas cell repellant.

We present a novel extension of the use of PEO-like films by exploiting our finding that this material, even the most highly “non-fouling” form, is in fact capable of modestly adsorbing aqueous polylysine, a positively charged polypeptide that promotes cell adhesion. This finding is consistent with a recent report of a slight tendency of a plasma polymerized, PEO-like material to adsorb some positively charged species.²⁶ We further determined that adsorption was greatly enhanced with slight chemical alteration of the surface chemistry via a brief plasma oxidation. This simple treatment represents a more subtle method for tuning surface properties than previous modifications demonstrated for this PEO-like film. Furthermore, the plasma oxidation, when combined with the adsorption of polylysine, can be leveraged not only to enable direct cell attachment but also to mediate the immobilization of other molecular species that could not otherwise be immobilized to the surface of the film without chemical derivatization.

We have harnessed the interaction of polylysine with PEO-like films to develop a simple and yet versatile and high-resolution micropatterning scheme that requires only a single deposition of a blanket background of PEO-like film along with a single microlithographic step to create micron-scale adhesive regions to effectively restrict cell body attachment and strictly guide axon growth. This technique does not require complex chemistries, and the resulting patterned

film has extended shelf life in ambient air. Additionally, this process is compatible with standard microfabrication processes and therefore, cellular and subcellular scale micropatterns can be integrated with virtually any biosensor and microdevice. In this study, we present the results of specific tests conducted to evaluate the effectiveness and versatility of the patterning technique. Specific objectives were 1) to compare the adsorption of a few key molecular species on the PEO-like film; 2) to demonstrate the ability of immobilized polylysine to mediate the adsorption of these other species; 3) to assess the viability of primary neurons and their ability for neurite outgrowth on patterned PEO-like films; 4) to quantify the compliance of cultured neurons and their axons with respect to the cell adhesive and adjacent cell repellent patterns; and 5) to determine whether photolithographic processes resulted in any chemical changes to the surface of the PEO-like film.

2. Experimental Section

2.1. PEO-Like Film Deposition

We performed film deposition in a Plasma-Therm PK-12, parallel-plate plasma system using platens approximately 12 in. in diameter. During deposition, a mixture of 20% diglycol methyl ether ((CH₃OCH₂CH₂)₂O, or DEGDME, or “diglyme”) vapor in argon (Ar) was maintained in the chamber at a total pressure of ~20 mT. An RF generator (operating at 13.56 MHz) produced plasma at a constant power of ~1–2 W in a low temperature environment. Deposition was performed for about 20 min. on cleaned, polished pyrex glass, positioned on the lower, ground electrode.

2.2. Oxygen Plasma

Pyrex samples with deposited film were treated with oxygen plasma using a March Plasmod plasma system. Surfaces were treated with 20 W of oxygen plasma for 15 s. at ~1.3 T. The duration of the oxygen plasma was limited to avoid eroding the photoresist and distorting the lithographic pattern.

2.3. Contact Angle

The wettability of water on the PEO-like film was measured using a Kruss Contact Angle Measuring System. Contact angles were determined from magnified images of sessile drops of ~10 µL deposited on the film surface with a miniature syringe. Numerous drops were measured for each sample, and data represent an average of at least 10 measurements.

2.4. AFM Film Characterization

A Digital Instruments Nano-scope Dimension 3100 atomic force microscope was used with a cantilever probe in tapping mode to characterize the topography of the film surface and to determine film thickness via step height measurement.

2.5. XPS Analysis

X-ray photoelectron spectroscopy was performed by an SSI S-Probe Monochromatized XPS Spectrometer with a monochromatic Al K α X-ray small spot source (1486.6 eV) and a take off angle of 45°. For characterization of film composition, a broad survey spectrum (0–1000 eV) was performed using a spot size of 1000 × 250 µm². This broad spectrum permitted the quantification of the relative surface compositions of C and O species based on the C1 and O1 peaks. Additionally, high-resolution spectra using a spot size of 800 × 150 µm² were also compiled for the 278–294 eV range to elucidate the relative contributions from the C1 peak's individual components, which represented signals from carbon bonding with different atomic species. For each high resolution spectrum, the individual components were determined from fitting the total spectrum to known peaks at 285, 286.5, 288, and 289.2 eV using Gaussian-

Lorentzian fitting (XPSPEAK 4.1). To prevent interference from chemical species in the underlying substrate, film thickness deposited on samples used in the XPS exceeded 30 nm, so that all measurements were from molecules from the film. (The X-ray source from XPS penetrated the material to a depth of about 10–20 nm from the surface.)

2.6. Protein Adsorption

To quantify the adsorption of protein,²⁷ phosphate buffered saline (PBS) solutions (pH 7.2) containing 1) fluorescein-labeled polylysine (200 µg/mL), or 2) bovine serum albumin (BSA) (100 µg/mL), or 3) immunoglobulin G (IgG) (100 µg/mL) were incubated on both native and oxygen plasma-treated PEO-like film for 1 h each at room temperature conditions. After the incubation, the samples were washed with DI water and airdried. In addition, the ability of preadsorbed polylysine to immobilize IgG was determined in samples that were first incubated for 1 h with 200 µg/mL of unlabeled polylysine, washed and dried, followed by incubation of 100 µg/mL of fluorescein-labeled IgG for an additional 1 h. To provide a point of comparison, the adsorption of each of species (polylysine, BSA, and IgG) was also performed on bare cell culture glass (MatTek Cultureware). The level of fluorescence present on the substrate (both PEO-like film and plain glass) following the various incubations was quantified by observation under a standard inverted microscope (Nikon TE 2000) under 10x objective magnification using a FITC filter and illuminated by a 150 W Hg lamp (Optiquip). Images were collected via a Retiga Q-Imaging EXi, cooled CCD camera and recorded on a desktop PC operating Simple PCI Imaging software (Hamamatsu Corporation). Lamp illumination, camera exposure and gain settings were strictly controlled to ensure that different samples could be compared.

2.7. Fabrication of Micropatterned Surfaces

µ-Polylysine-Adsorption-on-Cell-Repellant (µPLACeR) patterning process. (Figure 1) To create the micropatterned surfaces, the PEO-like film was blanket deposited on 4-in. diameter pyrex wafers. The film-covered wafer was then exposed for 1 min to vapors of HMDS to promote photoresist adhesion. (The wafer was not heated prior to this treatment.) A 1.3 µm layer of I-line positive photoresist (OiR 10i) was spin coated on the wafer followed by a 90 s soft bake at 90 °C. Desired patterns are then exposed on the wafer using a GCA 6200 wafer stepper, 10:1 reduction. The exposed pattern was developed with I-line developer (OPD 4262) for 1 min, rinsed with DI water and blown dry. By using positive photoresist, areas that were intended to be cell adhesive were open and not covered by photoresist following the development step. These lithographically developed substrates were subjected to a brief treatment of oxygen plasma, and then incubated with a 200µg/mL solution of poly-D-lysine (Sigma-Aldrich, 70 000–150 000 MW) in PBS (pH 7.2) for 1 h, washed with DI water and then dried in air. This step coated the lithographically defined, plasma oxidized regions of the PEO-like film with polylysine and rendered these regions cell adhesive, while the remaining areas were still cell repellant. Following this step, the remaining photoresist was removed by a 5–10 min. immersion in heated photoresist stripper (Baker PRS-3000) followed by 2 min. of sonication in the same stripper. The substrates were then thoroughly rinsed in distilled water and then exposed by UV through water and air-dried. The “lift-off” patterned polylysine areas remained and served as cell-adhesive regions, while the adjacent regions of the native PEO-like film, which were protected by photoresist and thus not coated with polylysine were cell repellant.

2.8. Polylysine Micropatterned PEO-Like Films after Photolithography

Films that had undergone the entire photolithographic process, from photoresist application to development and stripping were characterized using XPS to determine whether these treatments altered the chemical composition of the underlying material. (On samples for XPS

analysis, the polylysine was not introduced to the surface.) In addition, the degree to which polylysine that was adsorbed to the PEO-like film withstood the photoresist stripping process was investigated by comparing the binding of fluorescently labeled poly-L-lysine (Sigma) to the film surface before and after stripping. Of interest was whether and to what extent the photoresist stripping treatment removed adsorbed polylysine.

2.9. “Piggybacking” of Other Molecular Species

While many molecular species do not adhere to any meaningful degree on the PEO-like film, the presence of precoated polylysine on lithographically defined patterns on the film can mediate the immobilization of other bioactive proteins by their association with only the micropatterned polylysine. For example, a PEO-like film substrate with micropatterned polylysine can be incubated with a solution of laminin or IgG to immobilize these species on the same micropatterned regions. This simple “piggybacking” of other molecules of biological interest greatly extends the potential utility of the present micropatterning method.

2.10. Neuron Cell Culture

To evaluate the effectiveness of the micropatterned substrates for neuronal cell culture, primary hippocampal neurons from embryonic day 15 (E15) mice were plated onto the micropatterned substrates. The neurons were obtained using established protocols.²⁸ Briefly, hippocampi were surgically removed from dissected brains of the E15 mice, and cells were isolated via titration and enzymatic digestion. Cells were plated directly onto the micropatterned substrates and maintained in Neurobasal media (Invitrogen) supplemented with B27 (Invitrogen) and GlutaMAX (Invitrogen).

In addition to hippocampal neurons, retinal ganglion cells (RGC) obtained from 7-day old mouse pups using established protocols²⁹ were also cultured on patterned substrates in which the extracellular matrix molecule laminin was immobilized onto polylysine patterns.

2.11. Cell Viability and Compliance to Patterns

To quantitatively evaluate the viability of neurons cultured on the polylysine PEO-like films and the degree of cellular compliance to the patterned geometries, hippocampal neurons were plated on a checkered pattern, consisting of alternating $60 \times 60 \mu\text{m}^2$ squares of cell adhesive (polylysine coated) and cell repellant (bare PEO-like film) regions. To determine cell viability, cultures were stained with Fluo-4 AM calcium (Invitrogen) indicator dyes. Viable cells will fluoresce with an emission maximum of 525 nm as the calcium indicator is retained only in live cells after cleavage by esterases. Cell numbers on the adhesive regions were counted and compared to the number of cells on an equivalent area of the repellant region. To identify dead cells, cultures were stained with propidium iodide nucleic acid stain. Dead cells are permeable to this dye, which enters the dead cell and fluoresces upon association with nucleic acids. The compliance of neuronal attachment to adhesive regions was evaluated using the checkered patterns, on which cell bodies on both the cell adhesive and cell repellant squares were counted and compared. The compliance of neurite outgrowth along micropatterns was also evaluated along narrow $2 \mu\text{m}$ wide lanes of adhesive material. For cells on micropatterns, an anti-tubulin antibody (anti-TUB 2.1, Sigma-Aldrich) was used to stain intact microtubules using established protocols.³⁰

3. Results and Discussion

3.1. Film Characterization

The PEO-like film, generated by plasma-induced polymerization of diglycol methyl ether, was deposited on planar substrates to serve as a nonfouling background to prevent cell attachment.

To render this film as nonfouling as possible, the plasma power was kept minimal at around 1–2 W.^{14,20} However, it is important to note that our process used constant plasma power, as opposed to a pulsed delivery of plasma power. It is understood that pulsed delivery of power tends to reduce damage to the molecular structure of monomeric precursor, though both power formats have been successfully used for formation of PEO-like films.^{14,16,20} It is therefore possible that using low power can minimize molecular damage from ion bombardment, even under continuous power. While we did not characterize the molecular structure (i.e., degree of cross-linking) of our film, which was deposited under continuous power, we did characterize the chemical composition to confirm that it could serve effectively as a cell repellent material in its native form. Concurrently, films whose surface properties had been tuned by plasma oxidation were likewise characterized to determine the resulting change in chemical composition associated with the enhanced adsorption of polylysine.

3.1.1. XPS Analysis—As the first step in characterizing PEO-like film, the chemical composition of the deposited PEO-like film was determined using X-Ray Photoelectron Spectroscopy. By comparing the C1 and O1 peaks from the broad survey scan, it could be determined that the stoichiometric ratio of oxygen to carbon (O/C) was approximately 0.5, corresponding closely to the stoichiometry in the precursor molecule as well as polyethylene oxide itself. The high-resolution scan of the C1 peak, spanning 282–292 eV, revealed the contributions from the different types of carbon bonds (Figure 2). This C1 spectrum consists of four peaks: a major component at 285 eV arising from C—C and C—H bonds; another important peak at 286.5 eV due to C—O bonds (ethers); and lesser peaks at 288 and 289.2 eV corresponding to C=O/O—C—O bonds and COOR(H) (esters and carboxyl) groups, respectively. Each high-resolution scan was fitted to these four peaks, and the individual contributions of each peak to the overall spectrum were determined from this fitting. For evaluating PEO-like character, the first two major components, corresponding to C—C/C—H and C—O moieties, respectively, and their relative intensities are the most essential factors. In our film, the peak corresponding to the C—O bonds, at 286.5 eV, accounted for around 65–70% of the intensity of the C1 peak, with most of the remaining fraction accounted for by the peak corresponding to the covalent C—C and C—H bonds, at 285 eV (Figure 2A). This proportion implied that the film material had a PEO character ranging from 65–70% among three different samples. A small contribution from the C=O and O—C—O bonds, at 288 eV, was also present. Finally, contribution from the fourth component, representing ester and carboxyl groups (COOR(H)), at 289.2 eV, was negligible in the native film.

In previous work, it was determined that low power (~1–2 W) plasma was the most desirable for creating a nonfouling film with chemistry and stoichiometry closely matching polyethylene oxide.¹⁶ By applying low plasma power in our PEO-like film generation and deposition recipe, the chemical characteristics of our film closely matched those of previously demonstrated, nonfouling films. With respect to the stoichiometric ratio of carbon to oxygen, and the relative proportion of carbon-based bonds, our film is chemically similar to nonfouling versions of the PEO-like material reported (for both pulsed and continuous).^{14–17} Previous work has shown that material of this chemical composition resists most protein adsorption and strongly resists cell attachment, rendering this PEO-like film an appropriate selection as a cell repellent background for our cell patterning method.

Film samples treated with oxygen plasma were also analyzed under XPS (Figure 2B). Since the XPS measurements are derived from 10–20 nm depths within materials, it was difficult to precisely quantify changes at the very surface. Nevertheless, it was found that the brief oxygen plasma treatment slightly diminished the apparent PEO character of the film from ~65–70% to ~55%, while the oxygen to carbon ratio (O/C) remained at around 0.5. The decrease in PEO character was accompanied by a substantial increase of the C1 peak at 289.2 eV (to contributing about 7% of the C1 peak), indicating a marked increase in the proportion of COOR(H) (ester

and carboxyl) groups (Figure 2B, arrow). While the nonfouling nature of the PEO-like material has been attributed to the prevalence of ether bonds (C—O—C), the addition of ester and carboxyl groups to the surface tends to encourage the adsorption of species from aqueous solution.^{15,20}

3.1.2. Contact Angle (Table 1)—Surface hydrophilicity was characterized by contact angle measurements. Contact angle on native films averaged 59.7° (SD = 1.8, $n = 36$), which closely matched the PEO-like films reported previously. This contrasted with contact angle averages of 43.5° (SD = 3.8, $n = 20$) for the underlying polished pyrex glass. Treatment with oxygen plasma, as described, resulted in modest initial decrease of the contact angle to around 43.9° (SD = 2.6°, $n = 14$). When exposed to air, the contact angle relaxed to 48.0° (SD = 2.4°, $n = 12$) after 2 h, then to 52.7° (SD = 3.9°, $n = 12$) after 2 days, and finally to 57.0° (SD = 2.7°, $n = 12$) after 4 days. When the treated film was kept immersed in DI water at room temperature, the contact angle remained low and only relaxed to 47.4° (SD = 1.9°, $n = 15$) after 4 days. Plasma oxidation of polymeric materials such as poly dimethylsiloxane (PDMS) has been widely applied in various applications to render surfaces more hydrophilic via the addition of oxygen-containing surface groups. Specifically, it is believed that the exposure to reactive oxygen ions results in the addition hydroxyl groups along the surface, imparting the surface with more negative charge.^{31,32} However, it has also been well documented that these changes in surface characteristics reverse when exposed to ambient atmospheric conditions either through conformational changes of the polymeric chains at the surface or migration of oligomers from the bulk to the surface. Similar mechanisms may be taking place within the PEO film, although the phenomenon for this material remains to be explicitly investigated.

3.1.3. Surface Roughness—AFM measurements were performed in tapping mode along the surface of the native film with a cantilever tip (Figure 3). Scanning was performed within 5 $\mu\text{m} \times 5 \mu\text{m}$ areas at four random locations on the film surface (Figure 3A). The deposited film was found to be smooth within a 2 nm range (Figure 3A,B), too small to exert any topographical influences on cell attachment and behavior. This surface smoothness was unchanged after the brief plasma oxidation (Figure 3B). This result confirms that the change in contact angle arising from the brief plasma treatment can be attributed predominately to change of surface chemistry and not to physical topography.

3.1.4. Deposition Rate—AFM measurements were also used to determine thickness of deposited films. Measurements indicated that a thickness of 31 nm was obtained with a deposition time of 35 min under the described processing conditions, corresponding to a deposition rate of nearly 0.9 nm/min. This information was used to guide film deposition on process wafers, and a film thickness of around 15–25 nm was shown to mechanically withstand all of the subsequent photolithographic processes.

3.2. Protein Adsorption

Although plasma polymerized, PEO-like films are generally considered to be nonfouling, few studies have explicitly evaluated the adsorptivities of various species from solution on the film's surface, and limited data is available primarily for BSA. Our study sought to evaluate the adsorption not only of BSA but also the adsorption of polylysine and IgG molecules, which are commonly used in cell culture. Polylysine in particular is a positively charged molecule that has a widely known tendency to adsorb to many types of surfaces. Adsorption on surfaces of the native and plasma tuned PEO-like film were compared.

3.2.1. Quantifying Direct Adsorption—Glass substrates on which the PEO-like film was blanket deposited were incubated with fluorescently labeled versions of polylysine, BSA, and IgG. These incubation tests showed that polylysine adsorbed to the native film, though to an

extent less than on plain cell culture glass (Figure 4A). In contrast, BSA and IgG did not appreciably adsorb to the PEO-like film, as their fluorescent signal remained close to the background level and was much less than their respective adsorptions on plain glass. PEO-like film substrates that had been treated with oxygen plasma (20 W for 15 s at ~1.3 T) immediately prior to the incubation showed a marked increase in the adsorption of polylysine, even exceeding the adsorption of this species on plain cell culture glass. However, the adsorption of the BSA and IgG was only slightly increased (Figure 4A, right panel). We postulate that due to the positive charge of polylysine, the increase in adsorption of this species was due to an increase in negative charge-bearing moieties on the surface of plasmaoxidized film. As with many materials, even a brief exposure to oxygen plasma, hydroxyl groups will be added to the surface, transiently increasing the density of negative charge, which can promote more adsorption of polylysine.^{31–35} Indeed, the XPS analysis of our PEO-like material showed a marked increase in carboxyl and ester groups on the plasma oxidized surfaces, which was accompanied by a change in surface energy as seen in the change in the decrease in water contact angles. Previous studies have in fact shown that surface charge and wettability do have a significant influence the adsorption of molecules to surfaces.³⁶

3.2.2. “Piggy-backing” on Polylysine—Since it is a common practice to use a species like polylysine to facilitate immobilization of other bioactive molecules, we likewise harnessed the adsorption of polylysine for this PEO-like material to bring about immobilization of other molecular species that would otherwise be largely repelled by the surface of the native film. As a demonstration, we incubated films with polylysine (unlabeled) in PBS solution then followed that with incubation with IgG-FITC in PBS. While IgG alone does not adsorb appreciably to the film surface, it adsorbs readily (Figure 4A) onto surfaces that had been precoated with polylysine. Previous studies have demonstrated that surfaces coated with polylysine present fundamentally different apparent properties and exhibit different surface energies.³⁷

3.3. Surface Patterning

Since polylysine adsorbed onto the surface of the PEO-like film, particularly after plasma oxidation of the surface, we developed the μ PLACeR process, a cellular micropatterning scheme that involves a single plasma-enhanced film deposition and a single photolithographic step to produce a substrate that simultaneously provided well-defined cell adhesive regions surrounded by adjacent, complementary areas that were cell repellant. Our method of micropatterning involved the conventional spin coating of photoresist directly onto the PEO-like film and the application of standard photolithography on this substrate. The patterned photoresist served as the geometric template by which the polylysine immobilization was subsequently patterned by “lift-off”, creating patterns with resolution down to 1 μ m (Figure 1B). Following the adsorption of polylysine, the photoresist was completely stripped with the heated PRS-3000 stripper, leaving patterned cell adhesive regions coated with polylysine, and bare PEO-like film serving as cell repellant regions. This part of the PEO-like film remained physically and chemically unaltered throughout the photolithographic process, as indicated by XPS analysis of films that had undergone photoresist application, exposure, development and stripping (Figure 2C,D). Meanwhile, on the cell adhesive regions, the patterned polylysine on the surface of film was not affected by the photoresist stripping treatment, as there was no measurable erosion in the intensity of fluorescently labeled polylysine (Figure 4B). This micropatterning process was easy to implement, and many copies of a patterned substrate were simultaneously produced.

3.4. Cell Culture

3.4.1. Cellular Viability and Compliance on Patterned Substrates—To provide a more quantitative measure of the health of hippocampal neurons maintained on our patterned

substrates, cell densities on these substrates were compared to densities on standard polylysine coated glass 3 days after cells were plated under identical conditions at ~ 650 cell/mm². After 3 days, both substrates supported neurons with extensive neurite outgrowth and fasciculation. Cell densities on patterned substrates were similar to those on plain glass, and cell bodies and neurites faithfully followed the patterned geometries. Also, on both PEO-like film and conventional polylysine coated glass, a small number of dead cells stained by propidium iodide, could be observed interspersed with the live cells. These cells were small and spherical and, even under bright field, appeared distinct from living cells, whose cell bodies were flattened and spread out with multiple neurites extending. At day 3 there were an average of 471 (SD = 98, $n = 8$) cells/mm² on plain glass substrate coated with polylysine, while on the checkered pattern, a cell density of 952 (SD = 264, $n = 12$) cells per effective mm² of cell adhesive area was present (Figure 5A). This higher cell density on the checkered pattern is possibly attributable to the migration of neuronal cell bodies from cell repellant to cell adhesive areas during the initial period following cell plating, although such migrations have not been explicitly observed. Consistent with this interpretation, however, is our finding of local increases in neuronal density along edges of cell adhesive regions bordering cell repellant regions (Figure 5B). These results indicate that micropatterns of polylysine deposited onto PEO-like films is a good substrate for neuronal attachment and growth.

3.4.2. Organizing Primary Neurons, Neurites, and Potential Neural Circuitry Using Micropatterned PEO-Like Films—

To quantitatively assess the degree of cellular and neurite compliance to the patterned substrates, hippocampal neurons, harvested from embryonic mice using standard protocol, were cultured on PEO-like films containing a variety of polylysine micropatterns. Within just one hour of plating, the association of neurons with cell adhesive patterns were already apparent. Cell bodies began to adhere almost immediately to polylysine coated areas, just as on polylysine coated glass typically used in conventional neuronal cell culture. Regions of bare PEO-like film were completely cell repellant to hippocampal neurons, and no adhesion of cells to this surface were observed. Compliance to the desired patterns as determined by counting the number of cells attached to the polylysine regions compared to the number of cells attached to an equally sized region of bare PEO-like film. The results showed that a very high degree of cellular compliance was achieved by the current micropatterning protocol. On 12 different samples, 3473 neurons were counted on cell adhesive polylysine containing regions, while only three neurons were found to be located in nominally cell repellant regions.

To assess the compliance of neurite extension on cell adhesive regions, 2 μ m wide lanes of polylysine were patterned to serve as conduits to guide axonal and dendritic outgrowth. While in the initial 1–2 days after cell plating, cell bodies can be observed to adhere weakly to these 2 μ m lanes, these neuron cell bodies subsequently detached over the course of two days. In contrast, the slender neurites extended along the narrow lanes, faithfully following the trajectory of these lanes (Figure 5C, Figure 6B – F), including curved lanes. There were a few exceptions to this compliance at sharp bends, where neurites often appeared to “cut the corners”. We believe that this reflects the fact that axons and dendrites do not adhere to their substrates continuously along their length but only at periodic locations where they develop adherent protein complexes.

Since neurons communicate with one another via their axonal processes, a potential use of neuronal micropatterning is the creation of well-organized neural circuitry on device surfaces. A commonly used geometry for patterning neurons is a square lattice configuration in which narrow lanes intersect at 90° angles. At these intersections, widened, circular cell adhesive regions are patterned to allow cell bodies to comfortably adhere, while neurites run along the interconnecting, narrow lanes. We applied this standard configuration with our patterning

scheme, and found that the neuronal cell bodies and neurites complied with this simple circuit geometry (Figure 5C, Figure 6B,C).

3.4.3. Compatibility with Conventional Immunodetection Methods and Fluorescence Optical Imaging

—An important requirement for a versatile cell micropatterning method for biomedical research and perhaps for use in devices as well is compatibility with conventional cell function characterization. Glass substrates containing polylysine patterns deposited onto PEO-like films permit immunodetection of cellular constituents using conventional antibody immunostaining methods typically used for cell culture. Furthermore, neurons and axons grown on micropatterned substrates can be observed using standard optical microscopy that is widely available in research laboratories (Figure 6D).

3.4.4. Shelf Life

—Another advantage to this current scheme is the persistence of biologically active micropatterns in ambient conditions. Substrates with micropatterned PEO-like films have been left at room temperature conditions for over one month and were subsequently found to elicit high compliance attachment and neurite outgrowth from hippocampal neurons (Figure 5F). With most other techniques, patterned substrates must be used within a few days of preparation. Molecular monolayers in particular can degrade quickly after they are assembled on a substrate and are often subject to hydrolysis in aqueous environment.

3.4.5. Cell Culture of Primary Neurons Using “Piggyback” Molecular Patterning

—While the micropatterned polylysine can be used directly to culture many types of neurons, other neuron types frequently require the presence of specific bioactive adhesion molecules to mediate attachment, survival, and neurite extension on a culture substrate. We demonstrated (in Section 3.2.2) that polylysine adsorbed on the PEO-like film facilitated the immobilization of other molecules that would not otherwise adhere to the film. We applied laminin, an important component of the extracellular matrix to a substrate with patterned polylysine. Laminin only adhered to the polylysine coated regions and not on the bare film. Subsequently, when retinal ganglion cells (RGC),²⁹ which require laminin for adhesion,^{38,39} were plated on these substrates, cell bodies only adhered along patterned regions, and neurites within the 2 μm lanes faithfully followed the lanes' trajectories (Figure 6G). No cells or neurites were found in the nominally cell repellant areas. While we did not explicitly quantify the immobilization of laminin, these results are consistent with the “piggybacking” of laminin along the prepatterned polylysine and demonstrated the utility of the present micropatterning scheme as a platform for the simple microscale immobilization of a variety of biologically relevant molecules.

3.4.6. Advantages of Our Micropatterning Method

—Our μPLACeR micropatterning scheme is superior to other conventional approaches to neuron and neurite patterning in several key respects. Our scheme combines both cell adhesive and cell repellant regions side-by-side on a culture substrate to produce a high compliance of neuron cultures for a variety of configurations. By comparison, conventional patterning techniques have not produced the same high compliance and must often contend with cells taking hold within regions outside of the desired patterns. Microcontact printing, for example, often does not provide an explicitly cell repellant material to help enforce compliance, though more recent developments have incorporated such provisions. Methods that provide enforcement via cell-resistant molecular monolayers, such as those based on poly ethylene-glycol (PEG), still exhibit a lesser degree of cellular compliance to the desired patterns.^{2,4,5} This is due to the fragility of molecular monolayers and the difficulty in producing close-packed and continuous coverage over an entire surface. In contrast, the plasma-polymerized films are robust material—usually many molecules deep—that reliably provide continuous coverage and in the case of the PEO-like material, is highly resistant to cell attachment and adsorption of many molecular species.

While our μ PLACeR scheme is not the first application of these plasma-polymerized PEO-like film for patterning cell position and growth, it is much easier to implement compared with previously reported schemes and appears to be the only use of this material for neuron patterning. Strategies for using plasma polymerized films have focused on creating adjacent patterns of cell repellant and cell adhesive surface on the same substrates; this has included the direct patterning of the film deposition,^{22,23} combining different film materials side by side,^{14,17} and selectively altering, or tuning, surface properties on desired patterns.^{15,25} To pattern bioactive molecules on PEO-like films, μ CP has been used successfully to stamp a variety of cell adhesion species onto this material. This dependence on μ CP to deliver these molecules is due to the highly nonfouling nature of these materials, which are widely recognized to resist adsorption of molecular species from aqueous solutions but appear to accept these species readily when dry.^{22–24} However, our work has established that species such as polylysine can adsorb to these PEO-like materials from aqueous solution. Our scheme therefore exploits and enhances this previously overlooked property of the plasma-polymerized PEO-like films. This use of adsorbed polylysine in solution is not merely easier to implement than μ CP, but can be used to produce robust, high-resolution, cell adhesive patterns on the PEO-like film and in high volume (as in wafer scale production). In addition to serving as a direct as a molecular substrate for cell culture, polylysine can also be used as a foundation to immobilize additional molecular species that can then support the growth of more specialized populations of neurons.

4. Conclusions

We have demonstrated a simple yet robust technique for creating high-resolution organization and micropatterning of neurons and their cellular processes in culture. Our μ PLACeR technique uses a nonfouling, poly ethylene oxide (PEO)-like film as a background material for a cell repellant culture substrate. The plasma polymerized PEO-like film confers several important advantages for patterning. The film can completely cover a substrate. It is robust and stable in both ambient air and in aqueous solutions. As a nonfouling material, it is highly cell-repellant, and when blanket deposited, renders the culture background highly resistant to cell attachment. Nevertheless, despite its nonfouling character, the material does selectively adsorb polylysine, a positively charged molecule that is widely used for mediating cell adhesion to substrates.⁴⁰ With subtle tuning of the surface chemistry of this film via plasma oxidation, this adsorption can be greatly enhanced even though the film's nonfouling properties with respect to other molecular species are only slightly diminished. Based on this interaction between polylysine and PEO-like films, we developed a micropatterning scheme for neuronal and other cell culture involving a single plasma-enhanced, film deposition step along with a single photolithographic step to create high-resolution, cell adhesive micropatterns of polylysine set against a cell repellant background. Primary neurons maintained on substrates patterned with this method were healthy and complied nearly perfectly with the lithographically defined patterns, and neurite growth remained restricted to narrow lanes, demonstrating that the patterning technique is robust and reliable. Moreover, the patterned substrates themselves could be stored for extended periods in ambient conditions without noticeable degradation in biological activity or cellular compliance to the micropatterns. This versatile micropatterning technique can be readily adapted for many applications including the creation of simple neural circuits and can be easily integrated with fabrication methods for various biomedical microdevices and biosensors. Naturally, while this work focused on neurons and their thin processes, it is likely that the μ PLACeR patterning technique can be applied to other cell types as well.

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Figure 1.

μ -Polylysine-Adsorption-on-Cell-Repellant (μ PLACeR) patterning process. (A) Process layout. To create polylysine micropatterns, a glass substrate (usually 4-in. pyrex wafer) was positioned on the lower, ground electrode of a parallel plate plasma system. In (1), process gas, consisting of 20% vapors of diglycol methyl ether ("diglyme") in Ar was introduced into the chamber at a total pressure of ~20 mT. An RF generator was used to induce a plasma using a power of approximately 1–2 W. Under these conditions, the diglyme molecules polymerized to form a PEO-like, solid material that deposited uniformly on the glass substrate (2). The substrate, after being blanketed with the PEO-like film then underwent standard photolithography. In (3), photoresist was spin coated onto the surface of the PEO-like film and then exposed by UV through a photomask containing the desired micropatterns. After the exposed photoresist was developed (4), the underlying film was opened in the UV exposed regions, while photoresist remained to cover the adjacent areas. The surface was then briefly treated with oxygen plasma to chemically modify the exposed areas of the film (4). This was followed immediately by incubation with polylysine solution to immobilize this molecule on the film surface (5). The remaining photoresist was then removed (6), leaving polylysine only in the desired regions to promote cell adhesion. This is the conventional "lift-off" patterning. The adjacent regions of the PEO-like film, which were protected by photoresist, preserved their nonfouling character and remained cell repellant. Thus, a cell adhesive pattern is surrounded by a stable, cell repellant surface. (B) Resolution test patterns (numbers indicate pattern size in microns). The images show fluorescently labeled polylysine (bright areas) adsorbed and patterned by photolithographic lift-off on the PEO-like film. Photolithography performed on the plasma polymerized PEO-like film was capable of producing features as small as 1 μ m lanes (lower panel is zoomed image).

Figure 2.

High resolution XPS analysis of the film surface quantified the proportion of various types of carbon bonding within the PEO-like material. The C1 (carbon) peak has four main contributions: at 285 eV(C₁), 286.5 eV(C₂), 288 eV(C₃), and 289.2 eV(C₄) corresponding to the different bonds. The native film, as deposited, contained a stoichiometric ratio of oxygen to carbon (O/C) of approximately 0.5. From the high resolution spectrum, (A), the PEO-like character was about 70%, given the ratio of C—O to C—C/C—H bonds. This film was found to be highly nonfouling and cell repellent. With brief plasma oxidation, (B), PEO character was diminished somewhat to about 55%, while the presence of ester and carboxyl (COOR/H groups) increased markedly (arrow). During photolithography, the native film was subjected to various solvent treatments. In (C), native film was subjected to HMDS treatment, photoresist coating and then stripping. This is the treatment that unexposed film is subjected to during the photolithography. To represent what happens to the film underneath regions where photoresist was exposed and subsequently developed away, in (D) the film was subjected to HMDS treatment, photoresist spin coating, UV exposure, treatment with developer solvent and photoresist stripping at the end. In both (C) and (D), the photolithographic processes did not

alter the chemical composition of the film nor the relative proportion of carbon-based chemical bonds.

Figure 3. AFM imaging of the surface topography of the native film's surface. In (A), a topographical mapping of a $5 \times 5 \mu\text{m}^2$ region shows that the surface roughness remains within a 2 nm range. This can also be seen in an arbitrary (but representative) linear trace across the surface, (B, upper). This degree of smoothness was unchanged even after the brief plasma oxidation (B, lower).

Figure 4.

(A) Adsorption of molecular species from aqueous solution. The PEO-like film permitted the adhesion of polylysine but not of BSA and IgG molecules. However, the presence of polylysine immobilized on the surface permitted the film to adsorb other molecules that it would otherwise be resistant to, such as IgG. (In the column labeled “PLL+IgG”, the preadsorbed polylysine was unlabeled, while the IgG was fluorescently tagged.) Treatment with oxygen plasma enhanced the adsorption of the polylysine to a level comparable or higher than on cell culture glass, while the adsorption of BSA and IgG only increased slightly. The right-hand panel is a close up of the BSA and IgG data plotted in the left-hand panel. On the charts, the thicker solid lines indicate the average level of adsorption on cell culture glass. The adsorption on glass

provided a point of reference for each species, so that the adsorption of each on the PEO-like film relative to its adsorption on glass can be compared. The thinner lines indicate the average of the data points for native PEO-like film and the dotted lines represent the average of the data points for plasma oxidized film. (B) The adsorption of polylysine on both native (left) and oxygen plasma-treated films (right) was not measurably eroded by the photoresist stripping process. (Note: The fluorescence scale, vertical scale, is not the same for the left and right plots.)

Figure 5.

(A) Cell (Hippocampal neuron) viability and compliance was evaluated on a patterned checkerboard with $60\ \mu\text{m}$ squares. Fluorescently labeled polylysine was used to mark the cell adhesive squares, where cell bodies attached and appear as a lighter background compared to the bare PEO-like film repellant regions. Viable cells have been labeled with Fluo-4 calcium indicator. Cell attachment is exceedingly rare on the adjacent areas containing bare PEO-like film. (None were encountered in this sampled region.). (B) Along edges of cell adhesive regions, a local increase in cell density is typically seen. This effect is possibly due to limited migration of neuronal cell bodies away from cell repellent regions toward the cell adhesive regions, although a definitive explanation for this effect remains to be determined. (scale bar)

200 μm) (C) An array of 70 μm -wide, circular cell adhesive regions interconnected by narrow, 2 μm -wide, 200 μm -long cell adhesive lanes. The circular regions supported the attachment and growth of neuronal cell bodies, while the interconnecting lanes served as conduits to direct the outgrowths of neurites. Compliance of both cell body attachment and neurite outgrowth was nearly perfect on these patterns.

Figure 6.

Process of polylysine deposition on PEO-like film was used to produce micropatterns of various shapes and configurations for neuronal cell body attachment and neurite outgrowth. In (A), straight lanes of 10 and 20 μm widths permitted neuronal cell bodies to attach as well as neurites to take hold and extend. Due to the proximity of the cell adhesive lanes, neurites can sometimes cross the cell repellent areas to make connections with neurites and cells on nearby lanes. (B) A grid pattern with “wells” (circular, cell adhesive regions, 70 μm dia.) and interconnecting lanes (200 μm long, 2 μm wide) were used to test the compliance of both the cell bodies and neurites. Cell bodies remained exclusively within the wells, while neurites extending from these neurons followed the narrow lanes. (C) A wider field of view (Upper:

bright field, Lower: fluorescent) of a series of wells connected by 200 μm long channels shows cell bodies restricted to the circular wells while neurites run faithfully within the channels. (D) Tubulin within axons extending on micropattern substrates can be visualized after using an anti-tubulin antibody and standard cellular immunolabeling methods and observation using conventional fluorescence optical imaging. (Upper: brightfield Lower: fluorescent) In (E), we can see a neurite following the contours of a circuitous lane. However, at sharp turns, the neurite can be seen to “cut corners” (arrow). The adhesive lane extends along the dotted line to the cell adhesive patch at right. However, the neurite, which originates from the adhesive patch, cuts this corner. Except for cutting sharp corners, neurites were highly compliant with the patterned lanes. (scale bar) 50 μm) (F) Micropatterned substrates stored for over 1 month in room temperature and atmosphere conditions remained bioactive, permitted highly viable cultures, and produced a high degree of cellular compliance similar to that of substrates used soon after production. (circular, cell adhesive regions, 70 μm dia.; lanes 200 μm long, 2 μm wide) (G) Example of molecular ‘piggyback’ in which polylysine is used to further immobilize the extracellular matrix molecule laminin. The successful micropatterning of laminin using this method was verified by analyzing the pattern of neurite outgrowth from retinal ganglion cells (RGC), which is known to be laminin dependent, and do not extend on polylysine alone. The neurites of RGCs were found to follow faithfully the original polylysine pattern. (scale bar = 200 μm).

Table 1

Contact Angle (SD) (deg) of the Native and Plasma Treated Films

	native	oxygen plasma treated			
		immediate	2 h in air	2 days in air	4 days in air
contact angle	59.7 (1.8)	43.9 (2.6)	48.0 (2.4)	52.7 (3.9)	57.0 (2.7)
n	36	14	12	12	15