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Microscale Plasma-Initiated Patterning of Electrospun Polymer Scaffolds

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Abstract

Microscale plasma-initiated patterning (μ PIP) is a novel micropatterning technique used to create biomolecular micropatterns on polymer surfaces. The patterning method uses a polydimethylsiloxane (PDMS) stamp to selectively protect regions of an underlying substrate from oxygen plasma treatment resulting in hydrophobic and hydrophilic regions. Preferential adsorption of the biomolecules onto either the plasma-exposed (hydrophilic) or plasma-protected (hydrophobic) regions leads to the biomolecular micropatterns. In the current work, laminin-1 was applied to an electrospun polyamide nanofibrillar matrix following plasma treatment. Radial glial clones (neural precursors) selectively adhered to these patterned matrices following the contours of proteins on the surface. This work demonstrates that textured surfaces, such as nanofibrillar scaffolds, can be micropatterned to provide external chemical cues for cellular organization.

Keywords

Extracellular matrix; micropatterning; nanofibers; laminin-1; glial cells; nerve regeneration

1. Introduction

Biomaterials have been employed in an effort to mimic the physical and chemical properties of complex tissues [1]. To address the multiple architectural and chemical issues associated with physiological environments, the use of synthetic matrices such as electrospun nanofibers has emerged. These engineered scaffolds with controllable degradation *in vivo*

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and nanoscale guidance can reduce tissue destruction by allowing cells to grow into self supporting tissues [2].

Recent work indicates that permissive nanofibrillar substrates alone are unlikely to promote full recovery following tissue damage [3,4] and that chemistry as well as the geometry are critical for proper function. Our group has recently established a significant practical advantage of utilizing immobilized growth factors; they are more potent when presented as contact molecules attached to nanofibers than when presented to cells as a soluble molecule [5,6]. This approach allows a more permissive substrate for cellular attachment and proliferation, but still lacks the key feature of orienting cellular growth, which is pivotal for directed tissue regeneration.

For directed tissue regeneration, micropatterning has been used to influence the attachment, migration, and orientation of cellular processes [7-10]. This technology has allowed the study of how cell cultures *in vitro* organize into complex, physiologically relevant structures. Research in this field has been divided in two main areas: physical and chemical patterning methods. Physical patterning methods are widely used to form microgrooves or microstructures on the surface to provide topological cues for cellular orientation [11-14]. One of the drawbacks of this patterning method is that it requires the physical modification of the surfaces or scaffolds, compromising their mechanical properties as well as possible desired geometries. In contrast, chemical patterning methods allow for the modification of substrates with a variety of signaling molecules, without compromising the bulk physical properties of the material.

Most efforts in the chemical patterning area have focused on modifying the surface of materials using different technologies such as: microcontact printing [15-17], self-assembled monolayers [18], dip-pen lithography [19,20], nanografting [21] and ink jet printing [22], amongst others. These methods have had success in the surface modification of smooth surfaces, but no or limited success in the modification of complex, textured surfaces.

Recent work performed by Khana *et al.* [22] on poly(ϵ -caprolactone) (PCL) electrospun scaffolds presents a printing process where a commercial thermal ink jet printer is used to functionalize the surface of the scaffold. Discrete and continuous gradients of proteins were obtained at the interface of the scaffolds using this printing technique. However, the feature sharpness at the micron-scale level was minimal, compromising the efficacy of the patterns to guide cells in a specific orientation.

Combinatorial approaches have also been employed to create micron-size features on electrospun nanofibers. Shi *et al.* [16] combined microcontact printing, electrospinning and photolithography to create patterns of aligned or random nanofibers. This group describes a method for printing electrospun nanofibers from a collector (polydimethylsiloxane (PDMS) stamp) onto another substrate, as well as a post-printing process that involves photolithography and ion etching techniques to form microarrays on the printed nanofibers [16]. This method seems to be promising for a number of applications in cell biology, but the post-stamping process to form the arrays of the fibers can be challenging and difficult to translate into potential implantable devices for *in vivo* experimental setups.

This paper focuses on creating and evaluating microscale patterns (i.e., stripes) of biomolecules on the surface of nanofibrillar matrices utilizing microscale plasma-initiated patterning (μ PIP) [23]. μ PIP is used to immobilize aqueous-based biomolecular inks into micron-scale patterns on biomaterial substrates. The μ PIP method (Figure 1) uses a PDMS stamp to selectively expose or protect underlying substrate regions from chemical and physical effects of oxygen plasma exposure. Preferential adsorption of the biomolecules onto either the plasma-exposed (hydrophilic) or plasma-protected (hydrophobic) regions

leads to biomolecular micropatterns. The elastomeric PDMS stamp adjusts to the contours of the nanofibers, creating a relatively tight seal and preventing plasma leakage into stamp-protected areas. Following plasma-exposure, the surface is then exposed to the biomolecules. This process offers a unique advantage. As μ PIP does not require the transfer of “ink” from stamp to surface (as with microcontact printing), the final surface features are not affected by ink adhesion/distribution on the stamp.

The ability to create bioactive micron-sized patterns on the surfaces of electrospun nanofibers was demonstrated in this study with laminin-1, a neuroactive biomolecule. Laminin-1 is an extracellular matrix (ECM) protein that gives support to neural precursor migration during development and recovery of damaged central nervous system (CNS) and has been implicated in playing a pivotal role in coordinating axonal movement [24,25]. Micropatterned nanofibers with laminin-1 may be useful in regenerative medicine applications to build a synthetic extracellular matrix to encourage guided axonal regrowth following laceration of the nervous system.

2. Materials and Methods

2.1. Polyamide Nanofibers

Randomly oriented polyamide (proprietary composition) nanofibers (median diameter of ~ 180 nm) were electrospun onto plastic (Aclar) coverslips by Donaldson Co., Inc. (Minneapolis, MN) as previously described [4].

2.2. Patterned PDMS Stamps

PDMS stamps were prepared by pouring the Sylgard 184 silicone elastomer kit (Dow Corning Corporation, Midland, MI) (10:1 w:w base to cross-linker) over lithographically created masters, previously described in detail [26]. Briefly, masters were created by exposing photoresist-coated silicon wafers through a photomask, producing a relief pattern on the silicon surface. The relief pattern consists of a series of raised, parallel lanes $30\ \mu\text{m}$ wide separated by $50\ \mu\text{m}$ spaces, over which the silicone elastomer was poured. After curing, the PDMS layer was peeled off the master, and the patterned regions were cut using a razor blade into $\sim 10\ \text{mm}^2$ stamps. To ensure that the ends of the channels would be unobstructed during plasma treatment, the stamps were further trimmed on all sides to remove the outer portions of the patterned regions.

2.3. Biomolecular Ink

Laminin-1 (Invitrogen, Carlsbad, CA) ink was prepared by diluting the biomolecule in phosphate-buffered saline (PBS, pH 7.4, MP Biomedicals, Aurora, OH) to a concentration of $50\ \mu\text{g/mL}$.

2.4. Microscale Plasma-Initiated Patterning

The general microscale plasma-initiated patterning procedure was previously described in detail [23]. Briefly, this method utilizes a striped, patterned PDMS stamp to preferentially expose the underlying substrate and protect it from oxygen plasma (Figure 1). The patterned PDMS stamp, consisting of parallel lanes $30\ \mu\text{m}$ wide separated by $50\ \mu\text{m}$ spaces, was placed into contact with the polyamide nanofibers, and the entire unit was exposed to oxygen plasma (March Plasma, Concord, CA) for 300 s at 50 watts and 680 mTorr. Once plasma-treated, the stamp was removed and the polyamide nanofibers were transferred to a tissue culture plate and immersed in $200\ \mu\text{L}$ of biomolecular ink for no more than 30 min at room temperature. Typically, substrates were immersed in ink within 60 s after plasma treatment to allow the biomolecules to adsorb onto the hydrophilic regions of the substrate. Following the 30 min incubation of the inks, the nanofibers were gently swirled in a beaker

of deionized water (~20 mL) for approximately 10 s to remove non-adsorbed biomolecules and then stored in PBS at 4°C.

2.5. Radial Glia Cultures (RG3.6)

Neurospheres prepared from E13.5 GFP rat embryo forebrains were dissociated by trypsinization after 2 days following the procedure described by Li et al., 2004 [27]. For immortalization, the primary neural stem cells were cultured on laminin-1 coated substrates for 2 days and infected with PK-VM-2 retrovirus as described by Li et al., 2004 and Villa et al., 2000 [27,28]. After 4–5 days in selection in 200 µg/mL G418 (Invitrogen), individual cells were cloned at limiting dilution in 96-well plates. Cells were incubated and expanded in 10 cm dishes containing RG3.6 culture media. Media consisted of Dulbecco's Modified Eagle's Medium (DMEM/F12 + Glutamax) (Invitrogen) supplemented with 25 mM glucose (Sigma-Aldrich, St. Louis, MO), penicillin/streptomycin (Invitrogen), 10 ng/mL FGF-2 (Invitrogen), 2 µg/mL heparin (Sigma-Aldrich) and 1× B27 (Invitrogen). Before seeding the RG3.6 on micro-patterned nanofibers, cells were grown into neurospheres for 3 to 4 days in RG3.6 media. Then the neurospheres were then incubated in 1 mL of trypsin in 25 % EDTA (Sigma-Aldrich) for ~ 1 to 2 min to form a dispersed cell suspension. RG3.6 cells were cultured onto unmodified 12 mm nanofiber-coated coverslips or nanofiber-coated coverslips micropatterned with laminin-1 at a density of 100,000 cells/well in 24 well tissue culture plates. Cells were incubated in RG3.6 culture medium for 24 h at 37 °C and 10 % CO₂. After the 24 h of incubation, cultures were fixed in 4% paraformaldehyde in PBS and stored at 4°C in PBS until stained.

2.6. Immunostaining and Imaging

Laminin-1 patterns were immunolabeled with a rabbit polyclonal antibody against laminin-1 (Sigma-Aldrich) (1:100 dilution overnight at room temperature) followed by a CY3-conjugated goat anti-rabbit secondary antibody (1:500 dilution for 1 h at room temperature). RG3.6 cells cultured on nanofibers were immunolabeled with a mouse monoclonal antibody against nestin (Millipore Corporation) (1:500 dilution overnight at room temperature) followed by a CY3-conjugated goat anti-mouse secondary antibody (1:500 dilution for 1 h at room temperature). All secondary antibodies were from Jackson Laboratories (West Grove, PA) and were diluted in PBS containing 0.3% Triton X-100. Images were captured using a Zeiss Axioplan microscope equipped with an epifluorescence illuminator.

2.7. Scanning Electron Microscopy (SEM) Analysis

SEM was used to acquire images of the nanofibers and RG3.6 cells seeded on nanofibers micropatterned with laminin-1 using the previously described conditions. Cells were fixed in 2.5% glutaraldehyde in PBS. Following fixation, samples were serially immersed for 10 min in each of the following ethanol solutions: 50%, 60%, 70%, 80%, 90% and 100% ethanol. After dehydration, samples were supercritically dried (EMS 850 Critical Point Drier, Electron Microscopy Sciences, Hatfield, PA) and mounted on aluminum studs to be sputter-coated with gold-palladium using a SCD 004 Sputter Coater (Bal-Tec, Liechtenstein). Cell morphology and alignment was observed on an AMRAY1830 I scanning electron microscope.

2.8. Guidance Measurements

Digital image processing methods using the Fast Fourier Transform (FFT) algorithm were used to determine pixel directionality from images of cells cultured on micropatterned nanofibers [29,30]. To achieve this transformation, a gray-scale image of RG3.6 cells was processed with the FFT function of the ImageJ (NIH) image-processing software. The pixel intensities attributed to the FFT image where summed along a straight line from the center to

the edge of the image at a specific angle θ (90°). The relative contribution of objects oriented into the 90° direction was quantified. An image with aligned pixels has higher pixel intensities along a specific direction, plotted as having a single peak resembling a Gaussian distribution (Figure 2A). However, a random image results in a pixel distribution independent of direction, plotted as a line with multiple peaks (Figure 2B). Pixel summing was performed using the ImageJ plug-in 'Oval Profile' (available at <http://rsb.info.nih.gov/ij/plugins/oval-profile.html>). The graphical illustration of the pixel directionality of the original image is obtained by plotting the summed pixel intensities between 0° and 180° .

3. Results and Discussion

3.1. Micropatterns on electrospun nanofibrillar surfaces

Dimension plays a critical role in cell culture conditions, influencing how cells proliferate, differentiate, age, and react to drug treatments [1]. Therefore, micropatterned electrospun nanofibers are expected to incorporate both the chemical and physical cues of a more physiologically relevant environment for cell culture *in vitro* and tissue engineering applications. Figure 3 demonstrates that the μ PIP technique generates sharp patterns on the surface of electrospun nanofibers. Micropatterned nanofibers with laminin-1 were visualized by labeling the proteins that adhered to the surface with an antibody against laminin-1.

The randomly oriented polyamide nanofibers (Figure 3A) have some inherent fluorescence due to its polymer chemistry, creating a background that appears as unspecific adsorption of laminin-1 onto the plasma-protected regions. This effect is more appreciable in lower magnification fluorescence images (Figure 3B), but in a higher magnification image (Figure 3C), the resolution of the laminin-1 patterns is evident. In Figure 3, the red stripes correspond to protein adhesion onto the hydrophilic, plasma-exposed regions for laminin-1. Stamps used to generate the laminin-1 patterns consisted of a series of raised, parallel lanes $30\text{ }\mu\text{m}$ wide (plasma protected, yellow arrow) separated by $50\text{ }\mu\text{m}$ spaces (plasma exposed, white arrow).

3.2. Cell organization on nanofibrillar surfaces

To determine cell organization, RG3.6 cells were seeded on nanofibers in the absence of signaling molecules and nanofibers micropatterned with laminin-1. RG3.6 cells have been proven to bridge spinal cord lesions and promote functional recovery following spinal cord injury [31]. Previous research with polyamide nanofibers demonstrated that neurite outgrowth is randomly oriented on unmodified nanofibers [32]. As the nanofibers are randomly deposited, cellular organization from the embryonic radial glia (RG3.6) presumably will be randomly oriented as well. This result was confirmed by visual evaluation of the epifluorescence image from cells grown for 24 h on unpatterned nanofibrillar matrices (Figure 4A). Immunolabeling of cultured cells with an antibody against nestin (red) showed no orientation preference, which is consistent with the randomly oriented nanofibers (Figure 3A). Pixel orientation analysis further validates a random orientation (Figure 4C).

Ample experimental evidence suggests that neuronal signaling molecules will be valuable in conjunction with synthetic biomaterials to promote axonal regeneration, guidance, and recovery of motor and sensory function following nervous system injury [6,32-34]. To examine the cellular orientation in the *in vitro* model, RG3.6 cells were cultured on the micropatterned nanofibers for a period of 24 h. Figure 4D shows the cellular orientation of RG3.6 cells seeded on micro-patterned nanofibers with laminin-1. After 24 h, immunolabeled RG3.6 cells (red) seeded on laminin-1 patterns were aligned following the

direction of patterns, exhibiting an elongated bipolar morphology. Further evaluation by guidance measurements (Figure 4F) showed high levels of orientation.

To further verify that RG3.6 cells seeded on laminin-1 patterns aligned following the direction of patterns, SEM analysis was performed. Figures 4B and 4E corroborated the data acquired via epifluorescent microscopy, which suggested a randomly oriented cellular outgrowth on unmodified nanofibers (Figure 4A) and an elongated bipolar morphology of the RG3.6 guided by the laminin-1 patterns on the surface of the nanofibrillar matrix (Figure 4D). This data reiterates the parallel organization between the direction of laminin-1 and the orientation of pattern-aligned radial glia.

4. Conclusions

This study demonstrates how a textured polymer matrix can be micropatterned to enhance cell orientation at the surface of polymer scaffolds to recreate the combination of physical and chemical cues exhibited by the ECM. High levels of alignment were obtained for single cultures of RG3.6 cells with micropatterns of laminin-1. This work also investigated larger patterns widths (50 μm stripes/30 μm gaps) to ensure that glial cells adhere to the micropatterns and create a highly ordered matrix for subsequent neuronal guidance. Matrix deposition by RG3.6 cells was highly oriented along the surface patterns proving that it is feasible to create well-defined cellular alignment on a textured surface.

Unlike other traditional chemical patterning techniques, μPIP generates well resolved micron-size patterns in the surface of nanofibrillar matrices. Overall cellular response to the patterns was very effective for the experiments performed. In this research, we demonstrated that patterning textured scaffolds, such as nanofibrous mats, provides new opportunity to integrate chemical cues for cellular orientation onto complex structures for directed tissue regeneration.

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Figure 1. Schematic outline of the μPIP method used to create biomolecular patterns on the surface of electrospun nanofibers.

Figure 2.

Diagram of digital image processing for Fourier transform analysis of pixel directionality for cell guidance measurements. (A) Pixel intensities summed in micropatterned nanofibers along a specific angle of 90° (90° = direction of the pattern) yielding a graph with a single peak resembling a Gaussian distribution. (B) Pixel intensities of nonpatterned nanofibers result in a pixel distribution independent of direction, which results in a graph with multiple peaks.

Figure 3.

(A) Representative SEM image of polyamide nanofibers, scale bar: 10 µm. (B) Epifluorescence image of polyamide nanofibers micropatterned with laminin-1 (red stripes). (C) Close up of epifluorescence image of polyamide nanofibers micropatterned with laminin-1 (Pattern size: 50 µm biomolecule stripes (white arrows) and 30 µm gaps (yellow arrows)).

Figure 4.

(A) Epifluorescence image of RG3.6 cells cultured on unpatterned polyamide nanofibers, scale bar: 200 μm. (B) SEM image of RG3.6 cells cultured on unmodified polyamide nanofibers, scale bar: 100 μm. (C) Fourier transform analysis of pixel directionality of the cell orientation in panel A. (D) Epifluorescence image of RG3.6 cells cultured on micropatterned polyamide nanofibers with laminin-1, scale bar: 200 μm. (E) SEM image of RG3.6 cells cultured on polyamide nanofibers micro-patterned with laminin, scale bar: 100 μm. (F) Fourier transform analysis of pixel directionality of cell orientation in panel D.