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Nebulized solvent ablation of aligned PLLA fibers for the study of neurite response to anisotropic-to-isotropic fiber/film transition (AFFT) boundaries in astrocyte–neuron co-cultures

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ABSTRACT

Developing robust *in vitro* models of *in vivo* environments has the potential to reduce costs and bring new therapies from the bench top to the clinic more efficiently. This study aimed to develop a biomaterial platform capable of modeling isotropic-to-anisotropic cellular transitions observed *in vivo*, specifically focusing on changes in cellular organization following spinal cord injury. In order to accomplish this goal, nebulized solvent patterning of aligned, electrospun poly-L-lactic acid (PLLA) fiber substrates was developed. This method produced a clear topographic transitional boundary between aligned PLLA fibers and an isotropic PLLA film region. Astrocytes were then seeded on these scaffolds, and a shift between oriented and non-oriented astrocytes was created at the anisotropic-to-isotropic fiber/film transition (AFFT) boundary. Orientation of chondroitin sulfate proteoglycans (CSPGs) and fibronectin produced by these astrocytes was analyzed, and it was found that astrocytes growing on the aligned fibers produced aligned arrays of CSPGs and fibronectin, while astrocytes growing on the isotropic film region produced randomly-oriented CSPG and fibronectin arrays. Neurite extension from rat dissociated dorsal root ganglia (DRG) was studied on astrocytes cultured on anisotropic, aligned fibers, isotropic films, or from fibers to films. It was found that neurite extension was oriented and longer on PLLA fibers compared to PLLA films. When dissociated DRG were cultured on the astrocytes near the AFFT boundary, neurites showed directed orientation that was lost upon growth into the isotropic film region. The AFFT boundary also restricted neurite extension, limiting the extension of neurites once they grew from the fibers and into the isotropic film region. This study reveals the importance of anisotropic-to-isotropic transitions restricting neurite outgrowth by itself. Furthermore, we present this scaffold as an alternative culture system to analyze neurite response to cellular boundaries created following spinal cord injury and suggest its usefulness to study cellular responses to any aligned-to-unorganized cellular boundaries seen *in vivo*.

1. Introduction

Greater understanding of the cellular changes in response to biomaterial topography has allowed for biomaterials to be developed that specifically alter cellular behavior to elicit more efficient tissue regeneration [1–3]. Several different modalities of biomaterials are used to examine glial or neuronal responses to micro- and nano-surface topographies. For example, astroglial cells attach more strongly to microfabricated pillars rather than to smooth substrates [4]. Polymer microchannels have been shown to induce hippocampal neuron polarization more so than immobilized nerve growth factor on smooth substrates [5]. Neural cell lines cultured on polymer nanowires induced these neurons to produce more neural markers in comparison to the neural cells cultured on smooth surfaces [6]. Aligned, electrospun fibers, another type of biomaterial topography, directed the extension of neurites [7,8] and

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helped mature Schwann cell differentiation [9]. Deciphering the mechanisms by which topography influences glial or neuronal behavior in manners supportive of regeneration will lead to better biomaterial technologies to repair the injured nervous system.

Of the topographical biomaterials stated above, aligned, electrospun fibers are most commonly used to mimic the anisotropic structural assembly of axons and glia in the uninjured peripheral nervous system [10] and within the white matter tracts of the uninjured spinal cord [11]. The ability of aligned, electrospun fiber topography to direct regeneration and recreate the anisotropic structure within the peripheral nerve or spinal cord is communicated clearly within recent *in vivo* studies [12–16]. In experimental models of spinal cord injury specifically, electrospun fiber topography was able to encourage a subset of astrocytes to migrate into an electrospun fiber-containing conduit instead of forming an astroglial scar [15]. These studies demonstrate that electrospun fibers have the potential to not only direct axonal regeneration, but also to direct the migration of astrocytes supportive of axonal regeneration.

While it is well established that aligned, electrospun topography has the ability to direct axonal regeneration within experimental models of spinal cord injury, aligned fibers also may be utilized to develop *in vitro* models able to recapitulate transitions from healthy tissue to injured tissue. Studies involving topographical biomaterial constructs present cells with uniform topography, and cellular responses to such topography are compared to separate cultures where cells are cultured on flat surface controls [15,17]. To our knowledge, there are no biomaterial constructs that possess both aligned, electrospun fiber topography and non-topographical features within the same construct. Such constructs would provide information on how cells behave on topographical to non-topographical interfaces within the same culture, or to create models that mimic injury environments where cells at the lesion interface are not provided with topographical cues.

One injury with an anisotropic-to-isotropic transition is spinal cord injury (SCI), specifically within the white matter tracts. Following SCI, the extracellular environment is drastically altered, leading to changes in the composition and organization of the extracellular matrix. Furthermore, the distribution and alignment of astrocytes at the lesion edge becomes unorganized [11,18]. Immediately following injury, astrocytes migrate to the lesion edge, become hypertrophic and elongated, and create a dense cellular construct (termed the glial scar) [11,18]. These reactive astrocytes at the lesion edge alter the extracellular environment by up-regulating axonal extension-inhibiting chondroitin sulfate proteoglycans (CSPGs) [11,16]. Spared and regenerating axons within the white matter tract then extend to the lesion edge where they become dystrophic [19] and are not likely to cross into the lesion site due to the presence of axonal inhibitors [20,21] and the lack of a bridging scaffold to direct axonal regeneration [22].

Changes in extracellular composition and cellular function are very dynamic following SCI. While *in vivo* rodent models can provide information representative of spinal cord injury within humans [23], the surgeries require exceptional expertise. Additionally, the studies are very time consuming. Therefore, biomaterial constructs possessing topographical and non-topographical domains with a subset of cells found within the spinal cord may be used to assess the efficacy of pharmacological agents or to understand changes in cellular physiology at anisotropic-to-isotropic transitions in a more efficient manner.

In this study, we created anisotropic-to-isotropic fiber/film transition (AFFT) boundaries within electrospun scaffolds depictive of structural changes that occur following white matter SCI using a nebulized solvent technique. Nebulization technologies have been employed in the development of many innovative materials, including the fabrication of nanofiber coatings [24,25], light-emitting electrochemical cell coatings [26], coating and patterning of films with proteins or other molecules [27,28], and cell patterning and implantation [29–31]. The present study expands on previous nebulization technologies by developing a nebulization technique that produces smooth, isotropic topographical regions in aligned PLLA fiber scaffolds. Nebulized chloroform is used to ablate PLLA fibers, creating AFFT boundaries in the PLLA scaffolds. Furthermore, we hypothesized that astrocytes or astrocytes and neurons in co-culture would respond to the scaffold differently depending on whether the cells interfaced with the scaffold in a region with anisotropic topography or with smooth, isotropic topography. Either primary rat astrocytes or co-cultures of astrocytes and dissociated rat DRG neurons were cultured on these scaffolds. Following four days in culture, immunocytochemistry was used to assess astrocyte alignment and ECM orientation differences between anisotropic and isotropic domains. In astrocyte/neuron co-culture experiments, neurite outgrowth in different regions on the scaffold was assessed to examine the ability of growing neurites to extend from anisotropic topographical domains to smooth, isotropic topographical regions.

2. Materials and methods

2.1. Creation of aligned electrospun fibers

To create the aligned, electrospun fibers used here, collection films were first prepared. These films were made via the dissolution of 4 wt.% PLLA (NatureWorks, grade 6201D, Cargill Dow, Minnetonka, MN) in a 50:50 wt.% mixture of chloroform (EMD Millipore, Billerica, MA) and dichloromethane (Macron Chemical, Center Valley, PA). Solution was mixed continuously for 2 h at room temperature until full PLLA dissolution was observed. Afterward, the solution was casted onto 15 × 15 mm, thickness #1 glass cover slips (Knittel Glass, Baunsenweig, Germany) and allowed to dry overnight at 25 °C.

To begin the process of electrospinning, 8 wt.% PLLA was dissolved at room temperature in a 50:50 wt.% mixture of chloroform and dichloromethane. This solution was then electrospun in accordance with procedures previously published by the Gilbert laboratory [8,32]. Fibers were spun using a syringe with a 22 g sharp-tip needle (Fisher Scientific, Hampton, NH), which was insulated to establish high electrical charge only at the needle tip. A variable-speed syringe pump from Razel Scientific (St. Albans, VT) was used to continuously pump the PLLA solution at a rate of 2 ml/h. The working voltage, supplied by a Gamma High Voltage Research power supply (Ormond, FL), was maintained at 10 kV. Fibers were collected onto collection films, which were attached to a spinning aluminum collection wheel (1000 rpm and a diameter of 15 cm) using double sided tape (3M, St. Paul, MN). During electrospinning, the following protocols were employed: distance between the spinning disk and the needle tip was 6 cm, fiber collection time was 20 min, ambient humidity and temperature was controlled between 32–45% and 18–25 °C respectively. For experiments where fibers were to be visualized using epifluorescence microscopy, 10 μg of rhodamine B (Sigma–Aldrich) was added to the electrospinning solution prior to fiber fabrication. Fluorescent fibers were kept isolated from light until cell seeding.

2.2. Creation of AFFT boundary electrospun fiber scaffolds

Smooth, isotropic topological domains were created within the PLLA fiber scaffolds with nebulized chloroform using an airbrushing technique (Fig. S1). First, two 7.5 cm × 2.5 cm glass microscope slides (Electron Microscopy Sciences, Hatfield, PA) were arranged parallel to each other. These slides were then separated by the width of 150 μm. Aligned, PLLA fiber samples were then placed 2 cm beneath the gap separating these slides, with fibers oriented perpendicular to the gap. This 2 cm distance prevented direct contact between fibers and slides, preserving fiber alignment. Chloroform was then airbrushed over the glass slides, selectively dissolving the fibers underneath the gap and creating smooth, isotropic topological domains within the aligned fiber scaffolds. After treatment with nebulized chloroform, fiber scaffolds were sterilized for 12 h at room temperature via ethylene oxide sterilization using 7-L liner bags and 5cc ampoules (Anprolene AN74i, Andersen Products, Haw River, NC).

2.3. Imaging and analysis of AFFT boundary domains and fiber alignment following nebulization

There was concern that nebulization may affect the alignment of fibers near the AFFT boundary. To analyze fiber alignment near the nebulization zone, scanning electron microscopy (SEM) was conducted. SEM micrographs were captured using a Carl Zeiss Supra55 setup with Direct Write Attachment. The fiber samples were attached to glass cover slips using conductive copper tape (3M), scaffold edges were secured to the copper tape using Pelco® Colloidal Silver Liquid (Ted Pella Inc., Redding, CA), and a Denton Desk IV Sputterer (Denton Vacuum, Moorestown, NJ)