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Varying the diameter of aligned electrospun fibers alters neurite outgrowth and Schwann cell migration

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ABSTRACT

Aligned, electrospun fibers have shown great promise in facilitating directed neurite outgrowth within cell and animal models. While electrospun fiber diameter does influence cellular behavior, it is not known how aligned, electrospun fiber scaffolds of differing diameter influence neurite outgrowth and Schwann cell (SC) migration. Thus, the goal of this study was to first create highly aligned, electrospun fiber scaffolds of varying diameter and then assess neurite and SC behavior from dorsal root ganglia (DRG) explants. Three groups of highly aligned, electrospun poly-L-lactic acid (PLLA) fibers were created (1325 + 383 nm, large diameter fibers; 759 + 179 nm, intermediate diameter fibers; and 293 + 65 nm, small diameter fibers). Embryonic stage nine (E9) chick DRG were cultured on fiber substrates for 5 days and then the explants were stained against neurofilament and S100. DAPI stain was used to assess SC migration. Neurite length and SC migration distance were determined. In general, the direction of neurite extension and SC migration were guided along the aligned fibers. On the small diameter fiber substrate, the neurite length was 42% and 36% shorter than those on the intermediate and large fiber substrates, respectively. Interestingly, SC migration did not correlate with that of neurite extension in all situations. SCs migrated equivalently with extending neurites in both the small and large diameter scaffolds, but lagged behind neurites on the intermediate diameter scaffolds. Thus, in some situations, topography alone is sufficient to guide neurites without the leading support of SCs. Scanning electron microscopy images show that neurites cover the fibers and do not reside exclusively between fibers. Further, at the interface between fibers and neurites, filopodial extensions grab and attach to nearby fibers as they extend down the fiber substrate. Overall, the results and observations suggest that fiber diameter is an important parameter to consider when constructing aligned, electrospun fibers for nerve regeneration applications.

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1. Introduction

Electrospun fibers are being developed as tissue engineering scaffolds because their geometric scale is similar to protein fibers within the native extracellular matrix (ECM) [1–3]. Several factors make electrospun fibers an attractive scaffold for tissue engineering. The technique of producing electrospun fibers is relatively simple, cost-effective and flexible [3–5]. Many different varieties of polymers have been electrospun as cell scaffolds [6,7] or drug carriers [8,9]. Multiple types of electrospun fibers such as random [10,11], aligned [12–16], and hollow or porous structures [17,18] are capable of supporting the attachment and proliferation of a variety of cell types. Furthermore, the geometrical properties of the fiber (such as its diameter) can be controlled by changing the polymer solution parameters [19] and processing conditions [20–22].

Compared to a smooth surface or a scaffold with random structures, aligned structures are capable of guiding neurite extension through a lesion site for regeneration within the peripheral nervous system (PNS) or spinal cord [23–28]. Aligned, electrospun fibers and aligned filaments direct in vitro neurite extension [12–15,25,29,30]. However, it is difficult to maintain a uniform fiber diameter within electrospun fiber samples. For example, neurites from rat embryonic day 15 (E15) DRG proceeded along the aligned poly-L-lactide fiber scaffolds where the mean diameter was 524 nm but fiber diameter ranged from 150 to 1540 nm [12]. Most commonly, nanoscale, aligned, electrospun fibers are constructed to examine their ability to foster directed neurite outgrowth. For instance, aligned fibers with diameters in the nanoscale range (400–600 nm) constructed from acrylonitrile-co-methylacrylate guided neurite outgrowth from rat DRG [25]. Neurites from E10 chick dorsal root ganglia (DRG) also extended down nanofibers

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constructed of poly-ε-caprolactone (PCL) and collagen/poly-ε-caprolactone (C/PCL) [30]. In another recent study, neurites from E10 chick DRG were guided along aligned nanofibers made from PCL and were not guided when introduced to crossed patterns [15].

While electrospun nanofibers have been more extensively studied than fibers with diameters in the micron range, electrospun fibers or polymer filaments with diameters in the micron range also effectively direct neurite extension. Postnatal day 1 rat DRG neurite extension was more prolific on subcellular size filaments (5 μm) than on large diameter species [29]. Neurites from E9 chick DRG extended along aligned fibers whose diameter was between 1 and 2 μm [14]. From examination of these studies, it was clear that neurites emanating from DRG on aligned nanofibers extended longer neurites parallel to the aligned fibers. However, in some images, neurite extension perpendicular to the aligned, electrospun fibers was also seen [12,15,30]. Thus, it may be possible to alter topographical properties (e.g. fiber diameter) to minimize perpendicular neurite extension and promote more effective, longer neurite extension along the aligned substrate.

Our previous research has shown that highly aligned fibers with diameters in the 1–2 μm range have great potential in guiding in vitro neurite outgrowth from DRG explants in a directed manner [14]. We hypothesize that the surface morphology of aligned, electrospun fiber scaffolds such as fiber diameter is an important parameter that affects cell attachment, cell migration, and neurite orientation and extension from explants. The objective of this study was to create highly aligned fiber scaffolds with distinct differences in fiber diameter. Once the fiber scaffolds were fabricated and characterized, E9 chick dorsal root ganglia (DRG) were cultured on the fiber scaffolds to study how neurites interact with the fibers and to examine the effect of fiber diameter on neurite extension and Schwann cell (SC) migration. This study shows that the direction and extent of neurite extension and SC migration from DRG explants is influenced significantly by fiber diameter.

2. Materials and methods

2.1. Preparation of aligned, PLLA electrospun fibers with different diameters

Poly-L-lactic acid (PLLA) fibers were fabricated using an electrospinning setup described in our previous study [14]. Briefly, three fiber groups with different diameters were fabricated in this study. To obtain the large diameter (1325 ± 383 nm) fibers, 8 wt.% PLLA (NatureWorks™; grade 6201D, Cargill Dow LLC, Minnetonka, MN) was dissolved in a mixture of chloroform and dichloromethane (50:50 wt.%) (Sigma–Aldrich, St. Louis, MO) at room temperature. 8 wt.% PLLA was dissolved in 1,1,1,3,3,3-hexafluoro-2-propanol (HFP, Fluka/52512, Sigma–Aldrich) which yielded intermediate (759 ± 179 nm) diameter fibers. The addition of 0.2 wt.% of 10× phosphate-buffered saline (PBS) (Invitrogen, Carlsbad, CA) into a PLLA/HFP solution, created the small diameter fibers (293 ± 65 nm). All fibers were electrospun with a 22 G sharp-tip needle (Fisher Scientific, Hanover Park, IL). The tip of the needle was insulated with a piece of tubing so that the electrical force would concentrate on the needle tip, and consequently enhance the electrospinning efficiency. The syringe pump flow rate for all experiments was fixed at 2 ml h^{-1}, and the working voltage was held constant at 15 kV. The fibers were collected on 15 × 15 mm glass coverslips (Proscitech, Australia), attached on the edge of a rotating disk (220 mm in diameter with a thickness of 10 mm) using a piece of double-sided tape (3M; St. Paul, MN). 4 wt.% PLLA was used to make a thin layer of film to maintain fiber position and alignment. With this concentration, the viscosity of the polymer solution is suitable to cast the film. The mechanical properties of the film are appropriate for manipulation into three-dimensional scaffolds for use in other studies. PLLA was dissolved in a mixture of chloroform and dichloromethane (50:50 wt.%), and the solution was then cast on a coverslip prior to electrospinning. A thin layer of film formed as the solvent evaporated. Detailed working conditions of electrospinning and other parameters are shown in Table 1.

2.2. Cell culture

E9 chick DRG were isolated in accordance with procedures approved by the Institutional Animal Care and Use Committee (IACUC) at Michigan Technological University. Fiber samples were sterilized with an ethylene oxide sterilization system (Andersen Sterilizers Inc., Haw River, NC) for 12 h prior to culturing. DRG were isolated using previously published protocols [14]. Briefly, 200 μl of neurobasal media was placed on a fiber specimen. Then ganglia were divided into halves and placed within the neurobasal media droplet onto fibers. The culture experiments were repeated three times using independently fabricated fiber samples. DRG were allowed to attach onto fibers for approximately 6 h in a tissue culture incubator (37 °C, 5% CO_2) before adding another 1.8 ml of neurobasal media (with L-glutamine, penicillin/streptomycin and B-27 (Invitrogen), supplemented with 50 ng ml^{-1} of nerve growth factor (NGF) (Calbiochem, La Jolla, CA)). The DRG were then incubated for 5 days, with media being exchanged every 60 h.

2.3. Immunocytochemistry

2.3.1. Neurofilament stain

After 5 days of culture, DRG samples were fixed with 4% (w/v) paraformaldehyde (Sigma–Aldrich) in PBS (Invitrogen) solution for 30 min. Samples were washed three times with PBS, and then blocked with a solution containing 2% normal goat serum (Invitrogen), 2% non-fat dry milk (TVC Inc., Brevard, NC) and 0.05% Triton X-100 (EMD Chemicals, Gibbstown, NJ) for 30 min. After washing three times with PBS, samples were incubated (37 °C, 5% CO_2) with rabbit anti-neurofilament (145 kD intermediate neurofilament) primary antibody (1:200 dilution) (Millipore, Temecula, CA) for 1 h, and then washed three times with PBS. The specimens were incubated with an Alexa Fluor 488 goat anti-rabbit secondary antibody (Invitrogen) for another hour and washed three times with PBS.

2.3.2. Schwann cell stain

DRG samples were fixed with 4% (w/v) paraformaldehyde for 1 h. Samples were washed three times with PBS, and then blocked with a solution containing 10% normal goat serum (Invitrogen), 2% bovine serum albumin (BSA, Sigma–Aldrich), 0.4% Triton X-100

Table 1
Electrospinning parameters for fabrication of aligned fibers with different diameters.

Fiber type	Fiber diameter (nm ± standard deviation)	Collector speed (rpm)	Solvent	Espin time (min)	Working distance (cm)
Small diameter	293 ± 65	3000	1,1,1,3,3,3-Hexafluoro-2-propanol (HFP) +0.2% 10× PBS	3	3
Intermediate diameter	759 ± 179	1500	1,1,1,3,3,3-Hexafluoro-2-propanol (HFP)	6	6
Large diameter	1325 ± 383	1500	Chloroform/dichloromethane = 50/50	10	6