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The design of electrospun PLLA nanofiber scaffolds compatible with serum-free growth of primary motor and sensory neurons

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Abstract

Aligned electrospun nanofibers direct neurite growth and may prove effective for repair throughout the nervous system. Applying nanofiber scaffolds to different nervous system regions will require prior in vitro testing of scaffold designs with specific neuronal and glial cell types. This would be best accomplished using primary neurons in serum-free media; however, such growth on nanofiber substrates has not yet been achieved. Here we report the development of poly(L-lactic acid) (PLLA) nanofiber substrates that support serum-free growth of primary motor and sensory neurons at low plating densities. In our study, we first compared materials used to anchor fibers to glass to keep cells submerged and maintain fiber alignment. We found that poly(lactic-co-glycolic acid) (PLGA) anchors fibers to glass and is less toxic to primary neurons than bandage and glue used in other studies. We then designed a substrate produced by electrospinning PLLA nanofibers directly on cover slips pre-coated with PLGA. This substrate retains fiber alignment even when the fiber bundle detaches from the cover slip and keeps cells in the same focal plane. To see if increasing wettability improves motor neuron survival, some fibers were plasma etched before cell plating. Survival on etched fibers was reduced at the lower plating density. Finally, the alignment of neurons grown on this substrate was equal to nanofiber alignment and surpassed the alignment of neurites from explants tested in a previous study. This substrate should facilitate investigating the behavior of many neuronal types on electrospun fibers in serum-free conditions.

Keywords

Nerve regeneration; Polylactide; Poly(lactic-co-glycolic acid); Axon; Orientation

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1. Introduction

Aligned electrospun nanofibers powerfully direct the growth of regenerating neurites in vitro, offering promise as scaffolds for peripheral nerve repair [1–4]. Additionally, nanofiber scaffolds may be useful in guiding regenerating neurons in the spinal cord and brain, as well as serve to differentiate and guide transplanted neurons and stem cells in nervous system lesions. However, the ability to develop this technology as a therapy for nervous system injury depends upon being able to manipulate many complicated parameters relating to the biology of the cell types involved and the design of the scaffold. For example, the nervous system contains highly stereotyped morphologies of neurons in the cerebellum, hippocampus and olfactory bulb [5], as well as over 100 subtypes of neurons in the cerebral cortex alone [6]. Even in peripheral nerve, the simplest system in which to study regeneration, there are both motor and sensory neurons with multiple types of nerve fibers each [7], as well as Schwann cells expressing phenotypes that independently regulate motor and sensory neuron regeneration [8]. Moreover, numerous design parameters for fibrous scaffolds need to be resolved, including the choices of fiber material, diameter and alignment. How to best use various growth factors, extracellular matrix (ECM) proteins and cell–cell adhesion molecules with the fibers, whether as coatings or electrospun into them, also requires extensive exploration. All of these parameters are likely to need fine-tuning for specific neuron and glia cell subtypes and specific nervous system regions, which will certainly require rigorous in vitro experimentation.

However, the present state of in vitro materials testing is inadequate to perform such experiments. Instead of using primary neurons harvested from the nervous system, most in vitro testing of biomaterials relies on the use of renewable cell lines [1,2,9–13] or, more rarely, primary cells grown in media supplemented with serum [1,3,14,15]. Both of these approaches have significant drawbacks. Neuroblastoma cells and transformed neural stem cells have neurites that extend using contact guidance and thus grow along fibers, but their neurites do not resemble true axons and dendrites as judged by standard morphological and immunocytochemical criteria [16]. Typically, these cells attach and grow readily on materials without the surface coatings required by primary neurons to adhere and survive [1,2]. Therefore, their use to predict behavior of primary neurons in a specific regeneration application is very limited.

Using primary neurons with serum also imposes experimental limitations. In media supplemented with serum, primary neurons cannot be cultured at densities lower than 300 cells mm⁻² without a glial feeder layer [17]. This high cell density assures contact among neurons immediately after culture, precluding the study of how nanofibers influence the growth and differentiation of individual neurons and stem cells. In primary central nervous system (CNS) culture, serum favors the proliferation of glia [17], which can quickly overgrow a substrate. Furthermore, serum contains growth factors [18–20], soluble fibronectin [21] and thrombin [22], which affect neurite outgrowth, obscuring the effects of growth factors and ECM proteins coated on or spun into fibers. Using serum prohibits the ability to study the behavior of neuronal stem cells, since controlling stem cell fate depends on using specifically defined serum-free media [23]. Finally, serum-free culture has become standard practice since the development of Neurobasal, a media that supports primary neuronal survival in low-density culture [17,24]. The ability to grow primary neurons on electrospun fibers in serum-free conditions would be a significant advance in biomaterials testing.

We have been successful in directing the regeneration of neurites from serum-cultured dorsal root ganglia (DRG) explants along nanofibers made of electrospun poly(L-lactic acid) (PLLA), a biocompatible degradable polymer. In these experiments, PLLA fibers

electrospun on the target wheel were anchored on a glass cover slip with adhesive bandage [1]. When we tried growing dissociated neurons in serum-free conditions using these substrates, the neurons died by 4 days in vitro (DIV). Thus, growing dissociated neurons on PLLA nanofibers requires refining this technology to better support neuronal growth in serum-free conditions.

The objective of this study is to design substrates that better support the growth of dissociated, low-density primary neurons on aligned electrospun PLLA nanofibers in serum-free conditions. We tested primary motor and sensory neurons since both of these neuronal types are present in peripheral nerve, and because loss of both motor and sensory neurons is common to neurological disability. In the course of this study, we had several goals: first, to identify and eliminate any source of toxicity, whether in the fibers or in the materials used to fasten them to the substrate; second, to construct substrates with highly aligned PLLA nanofibers that retain their alignment during culture, fixation and staining; third, to electrospin nanofibers that maximize the observability of cells, by keeping the cells in the same focal plane of a microscopic field of view; fourth, to determine whether the inherently poor wettability of PLLA nanofibers compromises neuron survival, and whether increasing wettability with plasma treatment would improve cell viability; and last, to determine whether the neurites of dissociated neurons follow fibers more closely than neurites emanating from explants grown in serum.

Our investigation resulted in a substrate design produced by electrospinning PLLA nanofibers directly on glass cover slips on which a film of poly(lactic-*co*-glycolic acid) (PLGA) had been applied. On these substrates, primary motor and sensory neurons survive, grow and extend neurites oriented along the aligned nanofibers, allowing the first serum-free culture of dissociated primary neurons on electrospun fibers.

2. Materials and methods

All materials were purchased from Sigma–Aldrich (St. Louis, MO) unless otherwise specified.

2.1. Electrospinning

PLLA with an inherent viscosity of 0.55–0.75 dl g⁻¹ was obtained from Birmingham Polymers (Birmingham, AL) and dissolved in chloroform to a concentration of approximately 4 wt.%. The apparatus used for electrospinning is depicted in Fig. 1. The polymer solution was delivered by a syringe pump KDS 100 (KD Scientific, New Hope, PA) with a plastic needle and metal tip, to which an electrode is attached (spinnerette). A voltage of 10 kV was applied by a high-voltage DC power supply (Hipotronics, Brewster, NY). The target wheel, constructed at the University of Michigan, is 25 cm in diameter and has a beveled edge 0.16 cm wide. The wheel was grounded to attract the charged polymer. A motor (Caframo Ltd., Warton, ON) allowed varying the rotation to effect fiber alignment. A 5 cm distance was maintained between the spinnerette and target wheel. The wheel was rotated at 285 rpm to produce fibers of high alignment.

2.2. Substrate coatings

All coatings were applied in a sterile, laminar-flow hood. Substrates plated with motor neurons were pre-coated with poly-L-lysine (50 µg ml⁻¹) for 1–2 h and then washed twice in sterile water. Substrates plated with sensory neurons were pre-coated with collagen I (100 µg ml⁻¹) overnight and air-dried.

2.3. Cell culture

All experiments were done in accordance with the NIH Guide for Care and Use of Laboratory Animals as approved by the University Committee on Use and Care of Animals.

2.3.1. Primary sensory neuron culture—Primary sensory neurons were cultured as has been previously described [25]. Briefly, dorsal root ganglia (DRG) were plucked from the spinal cords of embryonic day 15 (E15) Sprague–Dawley rats, placed in L-15 media and pelleted by centrifugation at 500 g. Cells were dissociated by incubating in 0.05% trypsin/EDTA for 15 min at 37 °C followed by gentle trituration with a serum-coated glass Pasteur pipette. Cells were collected by centrifugation and plated in culture medium. Neurobasal (Invitrogen) supplemented with 2% B27 (Invitrogen) was used as the culture medium with the following additives: 30 nM selenium, 10 nM hydrocortisone, 10 nM beta-estradiol, 10 mg l⁻¹ apo-transferrin, and 1× penicillin/streptomycin/neomycin (Invitrogen). L-Glutamine (2 μM) was added to culture media immediately before plating. Identification of cells as sensory neurons was confirmed by immunostaining with an antibody to calcitonin gene-related peptide (CGRP; Fig. 2B).

2.3.2. Primary motor neuron culture—Primary motor neurons were cultured as has been previously described [26]. Briefly, perineural membranes were removed from spinal cords of E15 Sprague–Dawley rats and the tissue chopped into 2 mm pieces. Cells were dissociated by incubating in 0.05% trypsin/EDTA for 15 min at 37 °C followed by gentle trituration for 1 min with a serum-coated glass Pasteur pipette. Motor neurons were isolated over 5.4% Optiprep in L-15 media by centrifugation for 15 min at 1000g. Motor neurons were collected from the top layer above the Optiprep, and glia from the pellet. Cells were washed in L-15 media, then re-suspended and plated in culture medium. Neurobasal supplemented with 2% B27 was used as the culture medium with the following additives: 2.5 mg ml⁻¹ albumin, 2.5 μg ml⁻¹ catalase, 2.5 μg ml⁻¹ superoxide dismutase, 0.01 mg ml⁻¹ transferrin, 15 μg ml⁻¹ galactose, 6.3 ng ml⁻¹ progesterone, 16 μg ml⁻¹ putrescine, 4 ng ml⁻¹ selenium, 3 ng ml⁻¹ β-estradiol, 4 ng ml⁻¹ hydrocortisone, and 1× penicillin/streptomycin/neomycin. L-Glutamine (2 μM) was added to culture media immediately before plating. Identification of motor neurons was confirmed by immunostaining with an antibody to choline acetyltransferase (ChAT; Fig. 2A). Both cell types were counted with trypan blue and the plating density determined from the number of live cells.

2.4. Immunocytochemistry

To visualize cells grown on PLLA nanofibers, cells were fixed in 4% paraformaldehyde at RT for 15–30 min. To block non-specific antibody binding, samples were incubated in 1% goat serum/1.25% BSA/0.05% Triton X-100 in 1× PBS for 30 min. Primary antibodies, rabbit anti-neurofilament 1:1000 (Chemicon, Temecula, CA), mouse anti S-100 1:100 (Sigma), goat anti-CGRP 1:200 (Abcam, Cambridge, MA) and goat anti-ChAT 1:300 (Chemicon) were diluted in 2.5% BSA and incubated with cells overnight at 37 °C. The next day cells were washed in 1× PBS and incubated in secondary antibody (1:200 in 1× PBS) at RT for 2 h. Prolong Gold (Molecular Probes/Invitrogen), an anti-fade agent with 4',6-diamidino-2-phenylindole (DAPI), was used to stain nuclei. A Nikon Diaphot/FRET system was used for the imaging of most samples.

2.5. Determination of cell viability

Cell viability was determined by fluorescent imaging of cells stained with fluorescein diacetate and propidium iodide [27]. Fluorescein diacetate stains live cells green while propidium iodide stains dead cells red. In all experiments, fields selected for counting were representative of survival on the entire substrate and had an area of 0.916 mm². The

percentage of cell survival was calculated as the number of live cells divided by the total number of cells.

2.6. Cell survival on candidate fastening materials

Several candidate materials used to fasten fibers to substrates were tested for toxicity, including adhesive bandage (Johnson and Johnson, Skillman, NJ) silicone glue (Silbi-one MED ADH 4300 RTV, Rhodia Silicones, Ventura, CA) and PLGA in ratios of 85:15, 75:25 and 50:50 (Birmingham Polymers, Birmingham, AL) dissolved to a concentration of 10% (w/v) in chloroform. A small amount of each material was placed in the center of a 22×22 mm² glass cover slip (VWR Scientific, West Chester, PA) and neurons were plated at a density of 50 cells mm⁻². Cell viability on and around the material was assessed by fluorescein diacetate and propidium iodide staining after 4–5 DIV. An average of seven fields per substrate was counted, with the average number of cells counted in a field equaling 109 ± 48 (standard deviation, SD). Data were analyzed by averaging survival percentages across several trials. Since survival on positive controls varied slightly between trials, the survival percentage of each field was normalized to the average percent survival on glass controls for that trial.

2.7. Construction of fiber substrates and solvent-cast films

Three designs of cell culture substrates for electrospun nanofibers were fabricated and tested. The first design was made by electrospinning fibers directly on cover slips taped to the rotating target wheel. On these substrates, nanofiber bundles were not fastened down. The second design was made by electrospinning the fibers on the wheel, removing them as bundles and gluing the ends of the bundles to cover slips using PLGA. The third design was fabricated by taping cover slips to the rotating target wheel, coating them with a wet stripe of PLGA, then immediately electrospinning the fibers on to the PLGA stripe, as depicted in Fig. 1B.

To make PLLA solvent-cast films, a thin layer of PLGA 85:15, 10% in chloroform, was applied to a glass cover slip and allowed to dry for 30 min. A layer of PLLA, 4% in chloroform, was then applied on top of the PLGA. The underlying PLGA ensured that the solvent-cast PLLA remained attached to the cover slip throughout cell culture.

2.8. Scanning electron microscopy

PLLA fibers were first coated with approximately 100 Å of gold/palladium by sputtering (Technics Hummer VI). Scanning electron microscopy (SEM) was conducted using an Amray 1000-B, operating in high vacuum at 5 kV.

2.9. Determination of fiber integrity during culture

It was noticed on some of the substrate designs that, after immersion into culture media and fixation, fibers detached from the surface and fiber alignment within the bundles deteriorated. To assay this, substrates of each platform design were soaked in phosphate-buffered saline. Substrate detachment was evaluated by gross visual inspection. SEM was used to evaluate architecture of the bundle upon detachment.

2.10. Measuring surface wettability using contact angles

Images of 5 µl droplets of water on surfaces were captured using a Sony CCD/RGB camera, with samples mounted on a stage and a light source illuminating the droplet. Images were obtained in 18% humidity and 74.5 F. The NIH Image 1.63 angle tool was used to measure the angle where the droplet edge is perpendicular to the fibers. The contact angle measurement was obtained by calculating the complement of this angle. Two substrates for

each condition were assayed, with six measurements from each sample obtained. Some electrospun fibers and solvent-cast films were oxidized for 30 s with an air plasma before use (SPI supplies, West Chester, PA), in order to increase wettability [28]. Additionally, some samples were pre-coated with poly-L-lysine for 2 h prior to contact angle measurement.

2.11. MN survival on fibers with varying wettability

Motor neurons were plated at a density of 50 or 100 cells mm^{-2} and stained with fluorescein diacetate and propidium iodide after 4 DIV. An average of seven fields per substrate was assessed, with the average number of cells counted in a field equaling 107 ± 29 (SD). Data presented are averaged across three trials and normalized to the survival on glass controls plated at 50 cells mm^{-2} .

2.12. Quantification of fiber and neurite alignment

Alignment of fibers and neurites was quantified as previously described [1]. SEM images of fibers and dark field images of neurites were taken digitally and analyzed using ImageJ (<http://rsb.info.nih.gov/ij/>). Square regions of 256 pixels per side on SEM images of fibers and 512 pixels on fluorescent images of neurites were selected and processed for fast Fourier transform (FFT). FFT images were colorized for publication using a lookup table in ImageJ. A MATLAB script (The MathWorks, Natick, MA) was written to perform the analysis. From the origin of the FFT image, intensities along radii at each angle, (at 1° from the positive x -axis to 360°) were averaged. The average was calculated in a region (ρ , ρ) between user specified low and high radius values. For SEM images of fibers, ρ was chosen between 25 and 50 pixels in the FFT, corresponding to a range of 3.17–6.33 μm in the SEM image. For fluorescence images of neurites, ρ was chosen between 50 and 75 pixels in the FFT, corresponding to a range of 910–455 nm in neurite images taken with a $10\times$ objective and a range of 455–228 nm in the images taken with a $20\times$ objective.

Average intensities and corresponding angles were stored in an Excel spreadsheet. Average intensity was plotted against angle, generating two intensity peaks. For all data, a seven-point smoother was used on average intensity data to make the graph easier to interpret. The difference between angles on each side of the peak corresponding to one-half of the peak height, the full width-half maximum (FWHM), was calculated by averaging minimum and maximum intensities and locating the corresponding angle using the cursor, which caused the angle and intensity to be displayed. FWHM varied by $1\text{--}2^\circ$, unless ρ was made large enough to nearly envelop the entire FFT, in which case it usually decreased by $3\text{--}4^\circ$.

2.13. Data analysis

Statistics were calculated using Prism 3 software (www.graphpad.com). All data are presented as mean $\pm$ standard error of the mean. A one-way analysis of variance and Tukey's multiple comparison test were used to evaluate statistical significance.

3. Results

3.1. Neuron survival on fastening materials

We began our study by plating motor and sensory neurons on substrates of aligned fibers that had successfully guided the neurites of DRG explants in the presence of serum [1]. These substrates consisted of aligned fibers electrospun on the surface of a rotating wheel, peeled off and fastened to cover slips with adhesive bandage. However, when dissociated neurons were grown on these substrates in serum-free media, most cells were dead after 4 DIV (data not shown). We hypothesized that the adhesive bandage was toxic to dissociated primary neurons grown in serum-free conditions, resulting in extensive neuron death. To test

this, we compared the survival of E15 motor neurons grown on cover slips having a small square of adhesive bandage to survival on control glass cover slips. In addition to the adhesive bandage, cell survival was tested on other candidate fasteners, including a silicone glue and three different compositions of PLGA varying in the percentages of lactate and glycolate.

Motor neuron survival on cover slips with an adhesive bandage was substantially inferior to glass controls and the alternative fasteners (Fig. 3A). In four experiments, motor neuron survival decreased by an average of 39% in cultures with adhesive bandage. Survival was worst in fields just adjacent to the bandage, in some instances producing a complete loss of viable neurons (data not shown). Survival of motor neurons cultured on or around silicone glue and all compositions of PLGA was equal to glass.

Survival of E15 sensory neurons and Schwann cells was also measured on these candidate fastening materials by dissociating DRG harvested at the same time as the motor neurons (Fig. 3B). Compared to glass controls, adhesive bandage decreased cell survival in these experiments by 28%. This difference was also significant compared to PLGA 85:15, but not to the other PLGA compositions. Silicone glue, however, killed nearly all of the cells plated near it, reducing survival by 94% compared to glass. Survival on silicone glue was significantly less than all other fasteners tested. Similar to motor neurons, all compositions of PLGA produced survival comparable to glass controls.

3.2. Candidate substrate designs for aligned electrospun PLLA nanofibers

Since adhesive bandage and silicone glue were toxic to these neurons, and therefore incompatible with their primary culture, we designed several alternative substrates using non-toxic PLGA (85:15) to anchor fibers. The three designs we tested were made by (i) directly electrospinning the fibers on glass cover slips taped to the rotating wheel without the use of an adhesive material (Fig. 4A and B); (ii) electrospinning the fibers on the wheel, removing them and gluing the ends of the fiber bundles to cover slips with PLGA (Fig. 4C and D); and (iii) directly electrospinning the fibers on cover slips taped to the wheel bearing a wet stripe of PLGA at the onset of spinning (Fig. 4E and F). In this last design, fibers are deposited on the stripe as it dries. As the fibers accumulate on the stripe, they are anchored to the underlying PLGA, which in turn adheres to the underlying cover slip. Later versions of this design included the addition of a PLGA barrier along the perimeter of the cover slip to retain the cell suspension at the time of plating. All of these approaches produced very well aligned fibers, as examined by SEM.

3.3. Maintenance of fiber bundle integrity

To test whether fibers would detach from their cover slips, the three designs were submerged in PBS. Fibers that were spun directly on cover slips without a fastener (Fig. 4A) usually detached. If detachment occurred immediately or in the first few minutes, the fibers floated to the surface of the media as intact bundles. As observed with the unaided eye, these fiber bundles appeared intact with normal fiber alignment, but were dry on the top of the bundle where the cells would land during plating, which would be incompatible with cell viability. In other cases, fiber bundles remained submerged but became twisted as they floated, disrupting fiber alignment (Fig. 5A and B).

The other two designs performed better. Those fibers glued with PLGA at the ends of the bundle (Fig. 4C) also detached, but with less frequency than those directly spun without PLGA. These fiber bundles remained submerged, and became twisted only occasionally. However, visibility of cells after immunostaining was poor on these substrates as the bundle did not maintain a completely flat shape. Frequently, the cells failed to be in the same focal

plane in a microscopic field of view. Substrates on which fibers were electrospun on wet PLGA (Fig. 4E) also detached from the underlying glass and remained submerged. However, this substrate design maintained the best alignment, since separation occurred only between the PLGA stripe and the underlying cover slip. The fiber bundle and underlying PLGA detached as a unit and, although flexible, fiber alignment was always maintained after detachment (Fig. 5C and D). In addition, visibility of cells after immunostaining was far superior on these substrates as they consistently maintained a flat configuration.

3.4. Growth of dissociated primary motor and sensory neurons on aligned PLLA nanofibers

Fig. 6 shows E15 dissociated motor and sensory neurons grown on aligned protein-coated PLLA nanofibers that were electrospun directly on glass cover slips (Fig. 4E). These neurons were plated at densities between 50 and 200 cells mm^{-2} and grown in serum-free media for 4 DIV. Both motor neurons (Fig. 6A and B), isolated from E15 rat spinal cords, and sensory neurons (Fig. 6C and D), isolated simultaneously from DRG, oriented along aligned PLLA nanofibers. Similar to motor neurons cultured on glass or plastic [26], motor neurons on fibers had a long neurite, presumed to be an axon, and shorter neurites, which developed into dendrites. By 4 DIV, motor neurons had long axons, some of which extended the length of the photograph (approx. 450 μm). Some neurons were bipolar, but most had a multipolar morphology with an axon and multiple dendrites. Axons grew parallel to each other in close proximity, but remained separated and in general did not fasciculate. While axons sometimes turned and grew at an angle to the fibers for a short distance, they eventually returned to grow along the fibers for most of their course. Similarly, some dendrites appeared to initially grow radially away from the cell bodies before they turned to follow the fibers. At high magnification, individual motor neurons can be observed with clearly discernable axons and dendrites (Fig. 6B).

Sensory neurons were similarly oriented along the nanofibers and, when cultured at a higher density (100–200 cells mm^{-2}), showed processes that fasciculate and grow together, occasionally branching out to travel to another group of neurites (Fig. 6C). Numerous cells, indicated by blue-staining nuclei, were observed along the length of sensory neuron processes. These cells stained positively for S100, a marker for Schwann cells, which were also seen to traverse the fibers and appear to help orient the sensory neurons (Fig. 6D).

3.5. The effect of fiber wettability on primary neuron survival

Surface wettability is an important property of biomaterials affecting the attachment and viability of many different cells [29–31] including primary neurons [1]. Since PLLA nanofibers are very hydrophobic, we hypothesized that increasing their wettability (hydrophilicity) by exposure to an air plasma would increase cell survival. To determine the effect of plasma treatment on PLLA, we measured the contact angle of water on PLLA fibers and solvent-cast films, which served as a control for the topography of the PLLA fibers, as well as glass cover slips (Table 1). Since polylysine coating is required for motor neuron attachment, we also evaluated its effect on wettability. Plasma treatment significantly increased the wettability of both fibers and films. While polylysine increases the hydrophobicity of plasma-treated samples and glass controls, plasma-treated PLLA fibers and films are still significantly more hydrophilic than the non plasma-treated counterparts regardless of polylysine treatment.

Using polylysine-coated substrates, we then tested the effects of surface wettability on the survival of motor neurons plated at both 50 and 100 cells mm^{-2} . Contrary to our hypothesis, motor neuron survival was not improved by increasing surface wettability with plasma

treatment (Fig. 7). Motor neuron survival on plasma treated and untreated PLLA films were equal and similar to glass controls. Furthermore, plasma treatment actually worsened survival of motor neurons on fibers at the lower plating density of 50 cells mm⁻². This effect was not observed at the higher density of 100 cells mm⁻², suggesting that this is seen only at very low plating densities.

3.6. Quantification of fiber and neurite alignment

Nanofibers that were electrospun on PLGA-coated glass cover slips appeared to be very highly aligned. To quantify this observation, an analysis of fiber alignment was carried out in the frequency domain by first performing FFT of the spatial image (Fig. 8A) and then determining the value of the FWHM. The FWHM of the fibers was 20.6 ± 4.0 (mean $\pm$ SD) (Fig. 8F), a low value indicating very high alignment. To compare this fiber alignment to fibers spun on the wheel and taped to cover slips with adhesive bandage, results from our previous study [1] were analyzed against our present data (Table 2). No difference exists between the FWHM of our directly electrospun fibers and that of fibers spun on the wheel.

We then compared the orientation of neurites from dissociated motor and sensory neurons grown on nanofibers to the orientation of their neurites on planar substrates, which we hypothesized to be random. FWHM data were calculated for motor and sensory neurons grown on aligned fibers, as well as glass and solvent-cast PLLA, both planar substrates. Motor and sensory neurons grown on aligned nanofibers had FWHM values of 25.8 ± 4.7 and 29.4 ± 10.1 , respectively, indicating alignment equal to the underlying fibers (Fig. 8). Motor and sensory neurons grown on fibers were much more aligned than motor neurons (FWHM of 77.7 ± 26.4) and sensory neurons (FWHM of 59.9 ± 20.3) grown on planar surfaces. It should also be noted that, based on their FWHM values, sensory neurons grown on flat surfaces are more aligned than motor neurons grown on flat surfaces.

We also hypothesized that the orientation of neurites from dissociated neurons would be superior to that of neurites from DRG explants. In the data from our previous study [1], neurites from DRG explants had a significantly greater FWHM than the fibers they were grown on, indicating inferior alignment (Table 2). The significantly lower FWHM of dissociated neurons grown on fibers indicates that their neurites are more highly oriented than neurites emanating from the explants.

4. Discussion

Aligned electrospun nanofibers direct the growth of regenerating neurites, offering significant potential for their use in neural repair. The ability to grow dissociated primary neurons on nanofibers in serum-free culture is an important step in advancing the state of this promising technology. At the date of this writing, this is the first study to demonstrate the successful serum-free growth of primary motor and sensory neurons at low plating densities on aligned electrospun PLLA nanofibers (Fig. 6). This was achieved by developing a nanofiber substrate that met the following five criteria: (i) lack of toxicity; (ii) high fiber alignment; (iii) preservation of fiber alignment during culture, fixing and staining; (iv) maximized observability of cells; and (v) compatibility with plasma etching to allow testing of wettability.

The integrity of fibers and the viability of the cells depend on keeping the nanofibers organized and submerged in the culture media; therefore nanofibers must be securely fastened to a substrate. Obviously, this adhesive needs to be non-toxic. To fasten fibers, investigators have used adhesive bandage [1], biocompatible “super glues” [32], silicone glues [33] and silylated glass surfaces [3], as well as other fasteners. In this study, we found that PLGA proved to be a more biocompatible adhesive than bandage and silicone glue,

which proved to be toxic to primary neurons. We also tried super glue, which killed dissociated motor neurons (data not shown). Although bandage, silicone glue and super glue have been shown to be compatible with other cell types (e.g. transformed neurons) or when used with other media formulations (e.g. supplemented with serum), they are toxic to the neuronal types we tested in serum-free conditions.

Three considerations led us to choose PLGA 85:15 as the glue to fasten fibers. It is adhesive to glass, as are all three formulations of PLGA that we tested. Although we found that adhesion is greater with greater fractions of glycolic acid in the polymer (data not shown), PLGA 85:15 is the least autofluorescent of the three compositions, and it appears to promote better survival of sensory neurons and Schwann cells (Fig. 3). PLGA is also very easy to use as a fastener for nanofibers. Silylated glass is also compatible with serum-free culture of neurons [34,35] and can be used to fasten fibers [3], but mixing and applying PLGA to glass cover slips is faster and less complicated than silylation.

Of the different approaches we tested to fasten the nanofiber bundles to cover slips, PLLA directly spun on to a wet stripe of PLGA offers several advantages (Figs. 4 and 5). First, it retains alignment even after detaching from the cover slip. The other two designs (Fig. 4A and C), especially the one without PLGA glue, failed to retain their alignment upon detachment, precluding their usefulness in studying the growth of neurons on nanofibers. The second advantage is that handling of the fibers is simplified, since electrospinning directly onto the substrate obviates the need for collecting nanofibers from the wheel and then gluing them to a cover slip (Fig. 4C). Third, direct electrospinning onto the substrate, as opposed to electrospinning fibers on the wheel, produces a flatter fiber bundle. Cells cultured on relatively flat fiber bundles remain in the same focal plane, rendering them easier to observe and photograph during microscopy. Additionally, directly electrospun fiber bundles are dense after a few hours of electrospinning and are highly reproducible. This novel method of electrospinning on PLGA should be applicable to electrospun scaffolds made from other polymers.

The serum-free growth of primary neurons on aligned PLLA nanofibers shown in Fig. 6 is the direct result of our substrate design. Despite the toxicity of polylysine for in vivo applications [36], it is the standard substrate coating for primary CNS neurons in culture [37]. As such, it was critical that our nanofiber substrate could be coated with polylysine to promote primary motor neuron adhesion and growth. Although other proteins exist in vivo that can be used to promote cell adhesion (including the extracellular matrix glycoproteins laminin and fibronectin), polylysine produces greater physicochemical adhesion between cell and substrate. As it has no known direct integrin-mediated affect on neuronal differentiation, it is considered a more neutral substrate coating [38]. Since motor neurons in particular are very sensitive to environmental conditions and therefore difficult to maintain in culture [39–41], these results are promising and suggest that most neuronal types may survive and grow on this substrate design. Further experimentation with other types of neurons will be an integral step in elucidating the principles of neuronal behavior on nanofiber scaffolds and should lead to optimal designs that best promote regeneration in the nervous system.

In attempting to optimize cell survival and growth on PLLA nanofibers, we examined the role of wettability. Surface wettability affects cell behavior, with greater wettability promoting cell attachment [28], spreading [29], focal contact formation [42] and metabolic activity [30,43]. Wettability also affects survival of primary neurons, with more hydrophilic surfaces producing better viability [34]. Since PLLA is very hydrophobic, we hypothesized that increasing wettability of the fibers by plasma treatment would increase motor neuron survival. While plasma treatment increased surface wettability (Table 1), we found that it

did not improve neuronal survival, and that it even reduced the survival of cells plated at the lower density of 50 mm^{-2} . Since this effect was not observed on untreated fibers at the same plating density, we can conclude that plasma treatment alters the fibers in some way that reduces cell viability. It is plausible that plasma treating the fibers creates a toxin, since doubling the plating density, which increases the concentration of trophic factors, abrogates the effect. While viability was reduced on plasma-treated fibers, viability on plasma treated films plated at this density was equal to positive controls. Since nanofibers have a much greater surface area than flat films, plasma treating them could generate a higher level of toxin sufficient to produce cell death. Alternatively, since protein adsorption is decreased on more hydrophilic surfaces [44], plasma-etched fibers may adsorb fewer proteins, both secreted from cells and in the media, which are critical for cell survival. Another explanation relates to trophic support provided by synaptic connections with other neurons. While neurites on flat surfaces grow to connect with nearby neurons (Fig. 8C), neurites on nanofibers are frequently directed along the fibers past neighboring neurons (Fig. 8B). The low frequency of synaptic connection may deprive the cell bodies of the trophic support necessary to maintain their viability in the presence of a toxin.

Neurites grown on aligned nanofibers are highly oriented. In our previous study, we cultured DRG explants in serum on PLLA nanofiber bundles of varying alignment. We found that neurite alignment was dependent on nanofiber alignment, but that there appeared to be a limit in how closely neurites followed the fibers. Now that we can culture dissociated neurons on PLLA nanofibers, we wanted to examine whether neurites from dissociated neurons would orient on the fibers more closely than the neurites from DRG explants in our previous study. In an analysis of that data, we found that the FWHM of neurites from DRG explants cultured on highly aligned nanofibers was significantly greater than the FWHM of the nanofibers themselves, indicating that the neurites were less aligned than the underlying nanofibers. In contrast, dissociated motor and sensory neurons from the present study had a FWHM statistically equal to that of the nanofibers, indicating equal alignment. Dissociated neurons also had statistically smaller FWHM values than DRG explants, indicating superior orientation on the nanofibers.

There are several possible explanations for why neurites from dissociated neurons are more aligned than neurites from DRG explants. First, the number of neurites that grow from explants is profoundly larger than those from low-density sensory and motor neurons. This large number of neurites may simply “overwhelm” the capacity of nanofibers to orient them. Second, we had hypothesized that neurite fasciculation may decrease neurite alignment [1]. This could occur if neurites find other neurites a more attractive guidance cue than the underlying nanofibers. However, at the level of resolution of our photographs, dissociated sensory neurons from this study (Fig. 6C) show more apparent fasciculation than neurites from DRG explants [1]. After this observation, we believe that fasciculation may actually increase the alignment as measured by the FWHM. Third, serum in the DRG explant media may have an effect on neurite orientation along the fibers. Serum contains both proteins that potentiate neurite outgrowth, including IGF-I [19], laminin [45] and fibronectin [21], as well as non-adhesive proteins, such as serum albumin, that likely reduce neurite-to-fiber adhesion [46]. Therefore, serum could decrease neurite alignment by simultaneously potentiating rapid neurite growth on less adhesive nanofibers, encouraging neurites to jump from one nanofiber to another. The absence of serum in the media for dissociated neurons could explain their superior neurite alignment. Fourth, Schwann cells in culture underlie neurites and direct their growth [47]. If Schwann cells redirect elongating neurites from one fiber to another, then the large number of Schwann cells that migrate out from an explant may produce a cumulative effect on neurite pathfinding, reducing neurite orientation.

The ability to grow motor and sensory neurons in defined media on PLLA nanofibers profoundly expands the kinds of experiments that can be performed to optimize the use of nanofiber technology in nerve regeneration. We can now better characterize the influence of nanofibers on the differentiation and axonal pathfinding of primary neuronal types and neural stem cells, the impact of various fiber geometries on neuronal morphology and the effects of ECM proteins, their binding domains and growth factors electrospun into the fibers themselves. Using tools such as this to address critical questions about neuron–fiber interactions should allow investigators to design fiber scaffolds intelligently for a variety of neural regeneration applications.

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References

1. Corey JM, Lin DY, Mycek KB, Chen Q, Samuel S, Feldman EL, et al. Aligned electrospun nanofibers specify the direction of dorsal root ganglia neurite growth. *J Biomed Mater Res A* 2007;83A:636. [PubMed: 17508416]
2. Yang F, Murugan R, Wang S, Ramakrishna S. Electrospinning of nano/micro scale poly(L-lactic acid) aligned fibers and their potential in neural tissue engineering. *Biomaterials* 2005;26:2603. [PubMed: 15585263]
3. Schnell E, Klinkhammer K, Balzer S, Brook G, Klee D, Dalton P, et al. Guidance of glial cell migration and axonal growth on electrospun nanofibers of poly-epsilon-caprolactone and a collagen/poly-epsilon-caprolactone blend. *Biomaterials* 2007;28:3012. [PubMed: 17408736]
4. Chew SY, Mi R, Hoke A, Leong KW. Aligned protein–polymer composite fibers enhance nerve regeneration: a potential tissue-engineering platform. *Adv Funct Mater* 2007;17:1288. [PubMed: 18618021]
5. Peters, A. The fine structure of the nervous system. New York: Oxford University Press; 1991.
6. Molyneaux BJ, Arlotta P, Menezes JRL, Macklis JD. Neuronal subtype specification in the cerebral cortex. *Nat Rev Neurosci* 2007;8:427. [PubMed: 17514196]
7. Malcolm, B.; Carpenter, JS. Human neuroanatomy. Baltimore, MD: Williams & Wilkins; 1983.
8. Hoke A, Redett R, Hameed H, Jari R, Zhou C, Li ZB, et al. Schwann cells express motor and sensory phenotypes that regulate axon regeneration. *J Neurosci* 2006;26:9646. [PubMed: 16988035]
9. Dumont CE, Born W. Stimulation of neurite outgrowth in a human nerve scaffold designed for peripheral nerve reconstruction. *J Biomed Mater Res B: Appl Biomater* 2005;73B:194. [PubMed: 15660444]
10. Zhu X, Mills KL, Peters PR, Bahng JH, Liu EH, Shim J, et al. Fabrication of reconfigurable protein matrices by cracking. *Nat Mater* 2005;4:403. [PubMed: 15834415]
11. Li GN, Livi LL, Gourd CM, Deweerd ES, Hoffman-Kim D. Genomic and morphological changes of neuroblastoma cells in response to three-dimensional matrices. *Tissue Eng* 2007;13:1035. [PubMed: 17439391]
12. Yang IH, Co CC, Ho CC. Alteration of human neuroblastoma cell morphology and neurite extension with micropatterns. *Biomaterials* 2005;26:6599. [PubMed: 15936072]
13. Cui X, Lee VA, Raphael Y, Wiler JA, Hetke JF, Anderson DJ, et al. Surface modification of neural recording electrodes with conducting polymer/biomolecule blends. *J Biomed Mater Res* 2001;56:261. [PubMed: 11340598]

14. Goldner JS, Bruder JM, Li G, Gazzola D, Hoffman-Kim D. Neurite bridging across micropatterned grooves. *Biomaterials* 2006;27:460. [PubMed: 16115675]
15. Winter JO, Cogan SF, Rizzo JF III. Neurotrophin-eluting hydrogel coatings for neural stimulating electrodes. *J Biomed Mater Res B: Appl Biomater* 2007;81:551. [PubMed: 17041927]
16. Craig AM, Banker G. Neuronal polarity. *Annu Rev Neurosci* 1994;17:267. [PubMed: 8210176]
17. Brewer GJ, Torricelli JR, Evege EK, Price PJ. Optimized survival of hippocampal neurons in B27-supplemented Neurobasal, a new serum-free medium combination. *J Neurosci Res* 1993;35:567. [PubMed: 8377226]
18. Li Y, Fan J, Chen M, Li W, Woodley DT. Transforming growth factor-alpha: a major human serum factor that promotes human keratinocyte migration. *J Invest Dermatol* 2006;126:2096. [PubMed: 16691197]
19. Gu Y, Wang C, Cohen A. Effect of IGF-1 on the balance between autophagy of dysfunctional mitochondria and apoptosis. *FEBS Lett* 2004;577:357. [PubMed: 15556609]
20. Henrich D, Hahn P, Wahl M, Wilhelm K, Dernbach E, Dimmeler S, et al. Serum derived from multiple trauma patients promotes the differentiation of endothelial progenitor cells in vitro: possible role of transforming growth factor-beta1 and vascular endothelial growth factor165. *Shock (Augusta, GA)* 2004;21:13.
21. Grinnell F, Hays DG, Minter D. Cell adhesion and spreading factor. Partial purification and properties. *Exp Cell Res* 1977;110:175. [PubMed: 923661]
22. Turgeon VL, Lloyd ED, Wang S, Festoff BW, Houenou LJ. Thrombin perturbs neurite outgrowth and induces apoptotic cell death in enriched chick spinal motoneuron cultures through caspase activation. *J Neurosci* 1998;18:6882. [PubMed: 9712658]
23. Tropepe V, Hitoshi S, Sirard C, Mak TW, Rossant J, et al. Direct neural fate specification from embryonic stem cells: a primitive mammalian neural stem cell stage acquired through a default mechanism. *Neuron* 2001;30:65. [PubMed: 11343645]
24. Brewer GJ. Serum-free B27/neurobasal medium supports differentiated growth of neurons from the striatum, substantia nigra, septum, cerebral cortex, cerebellum, and dentate gyrus. *J Neurosci Res* 1995;42:674. [PubMed: 8600300]
25. Russell JW, Windebank AJ, Schenone A, Feldman EL. Insulin-like growth factor-I prevents apoptosis in neurons after nerve growth factor withdrawal. *J Neurobiol* 1998;36:455. [PubMed: 9740019]
26. Vincent AM, Mobley BC, Hiller A, Feldman EL. IGF-I prevents glutamate-induced motor neuron programmed cell death. *Neurobiol Dis* 2004;16:407. [PubMed: 15193297]
27. Jones KH, Senft JA. An improved method to determine cell viability by simultaneous staining with fluorescein diacetate-propidium iodide. *J Histochem Cytochem* 1985;33:77. [PubMed: 2578146]
28. Baker SC, Atkin N, Gunning PA, Granville N, Wilson K, Wilson D, et al. Characterisation of electrospun polystyrene scaffolds for three-dimensional in vitro biological studies. *Biomaterials* 2006;27:3136. [PubMed: 16473404]
29. Altankov G, Grinnell F, Groth T. Studies on the biocompatibility of materials: fibroblast reorganization of substratum-bound fibronectin on surfaces varying in wettability. *J Biomed Mater Res* 1996;30:385. [PubMed: 8698702]
30. De Bartolo L, Morelli S, Bader A, Drioli E. The influence of polymeric membrane surface free energy on cell metabolic functions. *J Mater Sci Mater Med* 2001;12:959. [PubMed: 15348348]
31. Lampin M, Warocquier C, Legris C, Degrange M, Sigot-Luizard MF. Correlation between substratum roughness and wettability, cell adhesion, and cell migration. *J Biomed Mater Res* 1997;36:99. [PubMed: 9212394]
32. Sun T, Norton D, McKean RJ, Haycock JW, Ryan AJ, MacNeil S. Development of a 3D cell culture system for investigating cell interactions with electrospun fibers. *Biotechnol Bioeng* 2007;97:1318. [PubMed: 17171721]
33. Yang F, Xu CY, Kotaki M, Wang S, Ramakrishna S. Characterization of neural stem cells on electrospun poly(L-lactic acid) nanofibrous scaffold. *J Biomater Sci* 2004;15:1483.
34. Corey JM, Wheeler BC, Brewer GJ. Micrometer resolution silane-based patterning of hippocampal neurons: critical variables in photoresist and laser ablation processes for substrate fabrication. *IEEE Trans Biomed Eng* 1996;43:944. [PubMed: 9214810]

35. Stenger DA, Hickman JJ, Bateman KE, Ravenscroft MS, Ma W, Pancrazio JJ, et al. Microlithographic determination of axonal/dendritic polarity in cultured hippocampal neurons. *J Neurosci Method* 1998;82:167.
36. Hunter AC. Molecular hurdles in polyfectin design and mechanistic background to polycation induced cytotoxicity. *Adv Drug Deliv Rev* 2006;58:1523. [PubMed: 17079050]
37. Banker, G.; Goslin, K. Culturing nerve cells. Cambridge, MA: MIT Press; 1998.
38. Lemmon V, Burden SM, Payne HR, Elmslie GJ, Hlavin ML. Neurite growth on different substrates: permissive versus instructive influences and the role of adhesive strength. *J Neurosci* 1992;12:818. [PubMed: 1545241]
39. Rakotoarivelo C, Petite D, de Weille J, Lumbroso S, Privat A, Sultan C, et al. Mild surfactation of neural cells, especially motoneurons, in primary culture and cell lines. *Exp Neurol* 2007;204:118. [PubMed: 17125768]
40. Arce V, Garces A, de Bovis B, Filippi P, Henderson C, Pettmann B, et al. Cardiotrophin-1 requires LIFRbeta to promote survival of mouse motoneurons purified by a novel technique. *J Neurosci Res* 1999;55:119. [PubMed: 9890440]
41. Ricart K, Pearson RJ Jr, Viera L, Cassina P, Kamaid A, Carroll SL, et al. Interactions between beta-neuregulin and neurotrophins in motor neuron apoptosis. *J Neurochem* 2006;97:222. [PubMed: 16524373]
42. Krasteva N, Groth TH, Fey-Lamprecht F, Altankov G. The role of surface wettability on hepatocyte adhesive interactions and function. *J Biomater Sci* 2001;12:613.
43. De Bartolo L, Morelli S, Bader A, Drioli E. Evaluation of cell behaviour related to physicochemical properties of polymeric membranes to be used in bioartificial organs. *Biomaterials* 2002;23:2485. [PubMed: 12033596]
44. Elbert D, Hubbell JA. Surface treatments of polymers for biocompatibility. *Annu Rev Mater Sci* 1996;26:365.
45. Burgmann H, Looareesuwan S, Viravan C, Vanijanonta S, Zedwitz-Liebenstein K, Vorbach H, et al. Serum laminin and basic fibroblast growth factor concentrations in patients with complicated *Plasmodium falciparum* malaria. *J Clin Immunol* 1996;16:278. [PubMed: 8886997]
46. Lochter A, Taylor J, Braunewell KH, Holm J, Schachner M. Control of neuronal morphology in vitro: interplay between adhesive substrate forces and molecular instruction. *J Neurosci Res* 1995;42:145. [PubMed: 8568915]
47. Thompson DM, Buettner HM. Oriented Schwann cell monolayers for directed neurite outgrowth. *Ann Biomed Eng* 2004;32:1120. [PubMed: 15446508]

Fig. 1.

Photograph of electrospinning apparatus. All components are pictured in A, including a DC power supply (1), syringe pump (2), syringe filled with PLLA solution and connected to an electrode (3), wheel with substrates attached (4) and a motor that rotates the wheel (5). A close-up of the wheel is pictured in B showing the grounding electrode (1), glass cover slips attached to the wheel with masking tape (2) and a PLGA stripe painted on to each cover slip (3).

Fig. 2.

Identification of E15 rat spinal motor and sensory neurons by immunostaining. In order to confirm the identity of cultured cells, motor neurons were labeled for ChAT (A) and sensory neurons for CGRP (B). Cells in the motor neuron culture stained positively for ChAT, which showed punctate expression in both the cell body and neurites. Neurons from dissociated DRG stained positively for CGRP, expressed in both cell body and neurites, confirming their identity as sensory neurons. All cells were stained with DAPI (blue). Scale bar = 25 μm .

Fig. 3.

Survival of E15 motor neurons and sensory neurons on candidate materials used to fasten aligned nanofibers to cover slips. Cells were plated at 50 mm^{-2} . Live neurons were stained with fluorescein diacetate (FD) and dead neurons with propidium iodide (PI). Survival was calculated as the number of live cells divided by total cells in a field. Each field was then normalized to the mean percent survival on glass controls. (A) After 4 DIV, motor neuron survival on bandage was decreased by 39% compared to glass controls. Survival on PLGA was similar to glass. (B) Example of FD-PI staining of motor neurons on PLGA 75:25. (C) After 5 DIV, sensory neuron survival was decreased by 93% on silicone glue and 28% on bandage compared to glass controls. Survival on PLGA was similar to glass. (D) Example of FD-PI staining of sensory neurons on PLGA 85:15. Data shown are averages of four trials for motor neurons and three trials for sensory neurons. * $p < 0.05$ and *** $p < 0.001$; error bars = standard error of the mean. Scale bar in (B) and (D) = $50 \mu\text{m}$.

Fig. 4.

Candidate substrate designs for aligned electrospun PLLA nanofibers. Photos (A, C, E) and SEM images (B, D, F) of nanofibers spun onto glass cover slips. (A, B) Fibers were directly electrospun on cover slips attached to the rotating target wheel. No glue was used to fasten them to the cover slip. (C, D) Fibers were electrospun on the rotating target wheel, removed, and glued to the cover slip at the ends of the fiber bundle with PLGA (85:15). (E, F) Cover slips were painted with a stripe of PLGA down the center and taped to the wheel, and PLLA fibers were electrospun while the PLGA was still wet. Additionally, a “moat” was created in this preparation by painting a wall of PLGA on the perimeter of the cover slip to hold the

cell suspension at the time of plating. SEM images reveal that fiber alignment is equal among each substrate design. Cover slips are $22 \times 22 \text{ mm}^2$, scale bar = $20 \text{ }\mu\text{m}$.

Fig. 5.

Preservation of fiber alignment after submersion. Substrates were immersed in PBS until fiber bundles floated off the underlying cover slips. (A) A fiber bundle that had been electrospun directly onto a cover slip without a fastener detached and floated in the media. The flat shape of the bundle was lost and the bundle twisted onto itself. (B) SEM photo of the same sample shows a small portion of the extensive loss of fiber alignment. (C) Fibers that are electrospun on wet PLGA retain their macroscopic architecture even after detachment, allowing handling throughout cell culture, fixation and immunostaining. (D) Alignment of the fibers is retained on the microscopic level, as seen in the SEM image. Scale bar = 20 μm .

Fig. 6.

Primary motor and sensory neurons (E15) grown on electrospun PLLA nanofibers in serum-free media. Nanofibers were coated with polylysine for motor neurons and collagen I for sensory neurons. In these images, fibers are aligned from left to right. Neurons are stained for neurofilament at 4 DIV (green). (A) Motor neuron processes are elongated and oriented parallel to the fiber bundle. Neurites are not perfectly aligned, as can be seen at the axonal branching point (arrow). (B) A higher magnification of a single motor neuron shows the alignment of the major neurite (arrow) and dendrites (arrowhead). (C) Similar to motor neurons, the long processes of sensory neurons are highly aligned and oriented parallel to the fibers. (D) Other cell types in the culture reveal similar alignment. Schwann cells labeled with S-100 (red) were seen closely associated with sensory neurites (arrowhead) and between sensory neuron processes (arrow). All cells were stained with DAPI (blue). Scale bar = 50 μ m.

Fig. 7.

Motor neuron survival as a function of surface wettability. Some samples were plasma treated to increase surface wettability. On polylysine-coated fibers and films cells were plated at a density of 50 or 100 mm⁻² and stained with fluorescein diacetate and propidium iodide at 4 DIV. Survival was calculated as the number of live cells divided by total cells in a field. Each field was then normalized to the mean percent survival on glass controls. Motor neuron survival rates were similar on glass, PLLA films and untreated PLLA fibers. Motor neuron survival was significantly decreased on plasma treated fibers at a cell density of 50 mm⁻². This decrease was not observed at a density of 100 cells mm⁻². Values are the average of three trials. **p* < 0.05 and ****p* < 0.001; error bars = standard error of the mean.

Fig. 8.

Quantification of fiber and neurite alignment. Representative images of fibers (A), motor neurons on fibers (B), motor neurons on flat surfaces (C), sensory neurons on fibers (D) and sensory neurons on flat surfaces (E) are shown alongside their corresponding FFT images. FFT images were produced by selecting representative squares 256 pixels on a side in SEM images and 512 pixels on a side in photomicrographs. FFTs were generated from the outlined regions as shown. Yellow to orange depicts high intensity of signal and magenta to blue depicts low intensity. The FWHM, a measure of the shape of the FFT image, was calculated using a MATLAB script for each sample. (F) Graph of the FWHM of fibers and both types of neurons grown on both fiber and planar substrates. Smaller FWHM values result from narrower FFT images and correspond to more highly aligned features in the spatial image. Fiber alignment and neurite alignment of both cell types on fibers were not statistically different from one another. Both motor and sensory neurons grown on fibers were more highly aligned compared to when grown on planar surfaces. Data shown as mean + standard error of the mean (n ranges from 19 to 41). *** $p < 0.001$, scale bar in A = 20 μm ; scale bars in B–E = 100 μm .

Table 1

Contact angle measurements of glass, PLLA solvent-cast films and PLLA fibers spun directly on PLGA stripe, with and without plasma treatment

	Contact angle		Significance
	Untreated	Plasma treated	
<i>PLLA film</i>			
–PL	59 ± 2.0	18 ± 5.1	<i>p</i> < 0.001
+PL	58 ± 4.2	39 ± 6.9	<i>p</i> < 0.001
<i>PLLA fibers</i>			
–PL	63 ± 10.5	32 ± 5.5	<i>p</i> < 0.001
+PL	68 ± 2.6	46 ± 4.9	<i>p</i> < 0.001
<i>Glass</i>			
–PL	27 ± 3.6		
+PL	43 ± 9.4		

Data shown are mean of 12 values ± SD. Plasma treating films and fibers significantly increases their wettability, making them as wettable as control glass. Coating with polylysine (PL) decreases wettability of plasma-treated samples but not the untreated samples. Despite the increase in contact angle, plasma-treated samples with polylysine remain hydrophilic relative to their untreated counterparts.

Table 2

FWHM of nanofibers and neurites in this study compared to previously published study of DRG explants on collagen-coated fibers in serum [1]

Study	Fibers	Neurites
Explants	23 ± 2	38 ± 6 ^a
Motor neurons	21 ± 4	26 ± 5
Sensory neurons		29 ± 10

Data are mean ± SD. FWHM of aligned nanofibers from both studies are equal.

^a Neurites from DRG explants have a significantly greater FWHM, indicating less alignment, compared to the nanofibers on which they grew ($p < 0.001$) and compared to the motor neurons ($p < 0.001$) and sensory neurons (from dissociated DRG, $p < 0.01$) tested in this study.