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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

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

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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., Wiarton, ON)

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