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Salicylic acid-derived poly(anhydride-ester) electrospun fibers designed for regenerating the peripheral nervous system

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Abstract

Continuous biomaterial advances and the regenerating potential of the adult human peripheral nervous system offer great promise for restoring full function to innervated tissue following traumatic injury via synthetic nerve guidance conduits. To most effectively facilitate nerve regeneration, a tissue engineering scaffold within a conduit must be similar to the linear microenvironment of the healthy nerve. To mimic the native nerve structure, aligned poly(lactic-co-glycolic acid)/bioactive polyanhydride fibrous substrates were fabricated through optimized electrospinning parameters with diameters of 600 ± 200 nm. Scanning electron microscopy images show fibers with a high degree of alignment. Schwann cells and dissociated rat dorsal root ganglia demonstrated elongated and healthy proliferation in a direction parallel to orientated electrospun fibers with significantly longer Schwann cell process length and neurite outgrowth when compared to randomly orientated fibers. Results suggest that an aligned polyanhydride fiber mat holds tremendous promise as a supplement scaffold for the interior of a degradable polymer nerve guidance conduit. Bioactive salicylic acid based polyanhydride fibers are not limited to nerve regeneration and offer exciting promise for a wide variety of biomedical applications.

Keywords

Nerve regeneration; electrospinning; fibers; polyanhydride; salicylic acid

1. Introduction

Traumatic peripheral nerve injury results in several hundred thousand operations in Europe and the United States each year in an attempt to alleviate neuropathic pain and repair functionality to innervated tissues.^{1,2} Although the peripheral nervous system is conducive for axonal regeneration, nerve entubulation is clinically preferred for directing regenerating axons and minimizing myofibroblast infiltration.³ To date, nerve autografts are commonly used to bridge peripheral nerve gaps, but are restricted by several issues: limited availability of nerves to use as an autograft, frequent functional loss at the donor site, mismatching nerve fascicles between the injured nerve and the nerve graft (size, length, and modality), and post-operative complications at the donor site (hyperesthesia or painful neuromas).^{4–8} A clear

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demand exists for ready-to-use nerve guidance conduits (NGC) available in a variety of pre-customized sizes and lengths to bridge and enhance nerve regeneration.

Limited clinical success has been achieved using hollow polymeric (both natural and synthetic materials) tubular nerve conduits for repairing peripheral nerve injury.⁹ The conduits facilitate nerve repair by bridging two injured nerve stumps and forming a protected lumen for the formation of a fibrin cable that enables Schwann cells to migrate from each severed nerve stump. The Schwann cells form longitudinally aligned strands (bands of Büngner) that guide axonal regeneration.¹⁰ Failure of nerve regeneration across long gaps (>15 mm in rats) is often the result of the initial fibrin cable not being able to form the connection from one severed nerve stump to the other.¹¹ Numerous studies have successfully proven that fibers are capable of directing both *in vitro*^{2,12,13} and *in vivo*¹⁴ peripheral neurite outgrowth. While most of these studies have guided axons with fibers that have diameters 100–250 µm in diameter,^{2,12–15} Wen et al. demonstrated that fibers with diameters at the size of a cell or smaller (5–30 µm), produce faster neurite outgrowth than fibers 100 µm in diameter or larger.²

Electrospinning is a well-established method for fabricating fibers with diameters ranging from nanometers to microns.¹⁶ A wide variety of polymers have shown to be capable of being electrospun, indicating that matrices with varying properties can be fabricated and customized for different applications.^{17–22} The electrospinning process involves applying a high voltage to a polymer solution which is slowly ejected from a syringe. Eventually, the electric potential at the surface charge surpasses a critical value and the electrostatic forces overcome the solution surface tension to form a thin jet of polymer solution. A complex interaction of variables (viscosity, surface tension, volatility, electrostatic forces, air friction, and gravity) cause the free end of the solution jet to follow a chaotic path as it travels toward a grounded collector.²² A grounded stationary planar collector results in a randomly oriented fiber mat, while a rotating mandrel or wheel target has the ability to align the fibers upon collection.²³ The biomedical functionality of aligned fiber constructs has been demonstrated with several types of cells (fibroblasts,²⁴ endothelial cells,²⁵ smooth muscle cells,²⁶ and chondrocytes²⁷) orientating parallel to aligned fibers. For the peripheral nervous system, previous research has demonstrated that regenerating axons follow winding, inefficient pathways, often resulting in misdirected re-innervation between sensory and motor axons and their targets.^{28,29} Aligned fibers within an NGC afford favorable topographical features for proficient glial and axon guidance from nerve stump to nerve stump.

Research has shown that fibers can be engineered from naturally occurring polymers such as collagen,³⁰ and several synthetic biodegradable polymers based on poly(lactic acid), poly(glycolic acid), or poly-ε-caprolactone (PCL).^{31–33} In this study, we electrospin admixtures of bioactive poly(anhydride-esters) and poly(lactic-co-glycolic acid) (PLGA) into either randomly orientated or aligned fiber mats with the goal of orienting glial cells and regenerating neurons within an NGC. Degradable polyanhydrides are utilized for a variety of biomedical applications and can be used as carriers of both physically admixed bioactive agents^{34–37} and bioactives that have been chemically incorporated into the polymer.^{38–40} Poly(anhydride-esters) are attractive because of their surface erosion degradation and pH-dependent, non-enzymatic, hydrolytic bond cleavage leads to the release of active therapeutics and water-soluble biocompatible byproducts. Poly(anhydride-esters) have high drug loading capabilities and drug release rates are controlled through a biocompatible linker.³⁹ The favorable physical and mechanical properties of the poly(anhydride-esters) from our laboratory allow for the fabrication of a variety of functional structures, including: films, disks, microspheres, conduits, and fibers.⁴¹

In particular, the poly(anhydride-esters) that are comprised of salicylic acid, the active component of aspirin,^{38–40,42,43} can hydrolytically degrade to release the non-steroidal anti-inflammatory (NSAID) (i.e. salicylic acid) and a biocompatible linker [Figure 1]. The NSAID is chemically incorporated into the polymeric backbone and has been shown to be therapeutically effective (anti-inflammatory, antiseptic, and analgesic).^{40,42,44} Using poly(anhydride-esters) comprised of salicylic acid as a drug delivery vehicle is advantageous because the polymer is a controlled drug delivery system with degradation products serving as therapeutic molecules. With a local delivery route, it may be possible to establish greater tissue-level concentrations of the drug at the desired target site than could be achieved using systemic treatment because of toxicity and absorption issues.⁴⁵

Locally releasing salicylate molecules from an NGC at the site of peripheral nerve injuries has exciting potential to interrupt the inflammatory cascade and alleviate various undesirable consequences (pain, swelling, glial scarring, and infection) without resorting to systemic NSAID therapy.⁴⁵ Additional on-going research is being performed to confirm and support the claim that the salicylic acid molecule is involved in activation and up-regulation of cyclin-dependent kinase – 5 (CDK-5), a cytokine involved in reorganization of the actin cytoskeleton during neurite outgrowth and regeneration.^{46–49} Encouraging research by Subbana et al. demonstrates that systematic administration of salicylic acid enhances functional recovery following a sciatic nerve crush in mice.⁵⁰

The result of this research is novel biocompatible polymer fiber mats that can be stacked within the lumen of an NGC. Aligned favorable fiber topography will guide neurite and glial outgrowth and migration with the additional capability of degrading into therapeutic components to synergistically enhance peripheral nerve regeneration.

2. Materials and methods

2.1 Polymer Synthesis

The salicylic acid polyanhydride (SAA) was prepared using a modification of a previously published method.⁴³ In brief, salicylic acid was dissolved in THF and pyridine. Adipoyl chloride was added dropwise to the stirring solution at room temperature. The reaction mixture was stirred overnight and then poured over water. After acidifying with concentrated HCl, the diacid was isolated by vacuum filtration, washed with water and dried. The diacid was activated by adding an excess of acetic anhydride until a homogeneous solution was observed. The acetic anhydride was removed under vacuum and the SAA polymer [Figure 1] was prepared by melt condensation of the monomer using the previously established polymerization conditions.⁵¹ The polymer obtained had a molecular weight of 14,000 Da and a glass transition (T_g) of 40 °C.

Poly(DL-lactide-*co*-glycolide) (PLGA) [50:50] (molecular weight ~45,000 – 70,000 Da as determined from an inherent viscosity of 0.76–0.94 dL/g in hexafluoroisopropanol, T_g of 46 °C), was purchased from Lactel Absorbable Polymers (DURECT Corporation, Pelham, AL).

2.2 Polymer solutions and electrospinning parameters

Following synthesis and characterization, the PLGA and SAA polymers were blended and placed in 20 mL scintillation vials and dissolved in a solvent mixture of dichloromethane (DCM) and dimethylformamide (DMF) [70:30].⁵² Solvents were purchased from Sigma-Aldrich (St. Louis, MO).

An initial screening of a wide array of polymers indicated that fiber fabrication was much more consistent with polymers that had relatively high M_w (> 30,000 Da) and with polymer-solvent solutions that were between 15–20% (wt/v). To improve the mechanical properties,

the salicylic acid-based poly(anhydride-ester) was blended with a higher molecular weight PLGA. Both randomly dispersed and predominately aligned fiber mats were fabricated by collecting the charged polymer jet on a grounded two-dimensional stainless steel plate and a grounded stainless steel rotating mandrel, respectively.⁵³

2.3 Electrospinning procedures

For the electrospinning process, each polymer solution was transferred from the 20 mL scintillation vials to a 10 mL syringe with an attached blunt 18-gauge stainless steel pipetting needle (Popper & Sons, Inc., New Hyde Park, NY). The syringe with the polymer solution (20% w/v) was placed on a custom made polyethylene stand in front of an abutting syringe filled with water and attached to an adjoining syringe on a syringe pump [Figure 2]. The distance from the stainless steel needle tip to the grounded collector was 10 cm and 5 cm for the stainless steel plate and rotating mandrel, respectively. A 20 kV charge was supplied to the stainless steel needle (18 G) via an alligator clip attached to a high voltage power supply (ES30P-5W/DAM, Gamma High Voltage Research Inc., Ormond Beach, FL). To obtain aligned fibers, a rotating mandrel set-up was utilized and consisted of a Dremel tool (300 Series, Dremel Division of Robert Bosch Tool Corporation, Racine, WI) clamped into a custom aluminum stand with a mandrel secured into a grounded stainless steel ball-bearing [Figure 2]. Not accounting for drag induced by the weight of the mandrel secured within the Dremel tool, all aligned fibers were collected at a setting correlated to ~5,000 rpm.

Both aligned and randomly orientated fibers were electrospun at a flow rate of 3.0 mL/h onto Reynolds non-stick foil. The fiber mats were easily removed and adhered to 12 mm glass coverslips (Fisher) with medical grade Secure Silicone Adhesive (Factor II Inc., Lakeside, AZ). Prior to being used, the glass coverslips were cleaned using Alconox (Alconox, Inc. NY) and H₂SO₄: H₂O₂(10:1, v/v) solutions and stored in 70% ethanol. After the fiber mats were adhered to the glass surface, they were placed in a dessicator under vacuum (Fisher, PYREX, England) for 24 h at room temperature. Coverslips were placed into 24-well plates (Fisher) and sterilized under UV-light at 254 nm for 900 seconds using the Spectrolinker XL-1500 UV crosslinker system (Spectronics Corp., Westbury, NY), immersed in 70% ethanol solution 900 s, and rinsed several times in phosphate buffer saline (PBS).

2.4 Fiber characterization

Fiber surface morphology was observed on an AMRAY 1830 I scanning electron microscope (SEM). Samples were mounted on aluminum studs and sputter-coated with gold-palladium using a SCD 004 Sputter Coater (Bal-Tec, Liechtenstein). SEM images were analyzed using ImagePro (Media Cybernetics, Bethesda, MD) software's linear measurement tool to assess fiber diameter and NIH ImageJ software (rsbweb.nih.gov/ij/) for fiber orientation.

Thermal analysis was performed on a TA Instrument DSC Q100 differential scanning calorimeter (Covidien, North Haven, CT). Thermal Advantage Universal Analysis 2000 software was used for data collection and processing. The glass transition temperature (T_g) was determined of electrospun fiber samples (4–8 mg) of both 2:1 and 1:1 PLGA:SAA ratios under nitrogen gas heating from –10 °C to 200 °C at a heating rate of 10 °C/min and cooling to 0 °C at a rate of 10 °C/min with two heating/cooling cycles. The T_g was calculated as half C_p extrapolated.

2.5 Drug release from the electrospun scaffolds (*in vitro* hydrolytic degradation)

Hydrolytic degradation of the polymers was performed by placing flat fiber mats (~1 cm²) in 20 mL Wheaton PET plastic scintillation vials (Fisher, Fair Lawn, NJ) with 10 mL of PBS and incubating them at 37 °C with agitation using a controlled environment incubator-shaker (New Brunswick Scientific Co., Edison, NJ) at 60 rpm for 56 days. Three samples for each electrospun polymer composition were analyzed. The PBS solution contained 0.1 M potassium hydrogen phosphate and 0.1 M potassium dihydrogen phosphate. The pH was measured with a pH meter (VWR Scientific, San Francisco, CA) and adjusted to 7.4 with 1 M sodium hydroxide and/or 1 N hydrochloric acid solutions.

For the first seven days of incubation, every 24 hours (every 7 days following the first 7 days) 1 mL of the buffer solution was removed and replaced by a fresh 1 mL aliquot of PBS. The spent media was analyzed by UV ($\lambda = 303$ nm) with a Perkin Elmer Lambda XLS UV/vis spectrophotometer (Perkin Elmer, Waltham, MA) to determine the release of salicylic acid. Measurements were taken at the maximum absorbance of salicylic acid that did not overlap with the UV absorbance of the adipic acid linker or salicylic acid diacid. A calibration curve was made from salicylic acid solutions of known concentrations and used to determine the salicylic acid concentration from the average UV data (n=3 per time point). Salicylic acid concentration values were corrected for the progressive dilutions at each sampling point.

In addition to measuring the release of salicylic acid from the electrospun fiber mats, the mass loss after the hydrolytic degradation was determined to confirm complete polymer degradation. The mass of the samples was determined using an AD-4 Autobalance (PerkinElmer). Samples were removed from the degradation media, placed in clean 20 mL scintillation vials, and washed in deionized water to dissolve any salts that the samples may have retained. The fiber mat samples were then lyophilized in a Freezone® 4.5 Freeze Dry System (Kansas City, MO) until the weight of the samples was constant and the dry mass of the samples was recorded.

2.6 Cell viability

Randomly orientated electrospun polymer scaffolds were further characterized by culturing NCTC clone 929 (strain L) mouse areolar fibroblast cells (ATCC, Manassas, Virginia) attachment and viability to the fiber surface. L929 fibroblast cells are a standard cell type for cytocompatibility testing as recommended by the ASTM.⁵⁴ As described above, the fiber scaffolds were sterilized in UV light and 70% ethanol once adhered to a coverglass surface via silicone adhesive. The fibroblasts were seeded onto the nanofiber scaffolds at a concentration of 25,000 cells/cm². Cell culture media consists of Dulbecco's Modified Eagle's Medium (DMEM, Sigma-Aldrich, St. Louis, MO), 10% v/v fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA), 1% L-glutamate (Sigma) and 1% penicillin/streptomycin (Sigma). Cell viability was determined by using calcein AM and ethidium homodimer-1 staining (Molecular Probes, Carlsbad, CA) and compared to a control surfaces of PLGA fibers and tissue culture polystyrene (TCPS). Cellular morphology was observed and documented at 10X original magnification with an Olympus epi-fluorescent microscope (Olympus, IX81, Center Valley, PA) at 24, 48, and 72 hrs hours post seeding.

Statistical analysis was performed with Minitab software (Minitab Inc; State College, PA) version 15.0 for Windows. For each of the three time-points (24 hr, 48 hr, and 72 hr), ANOVA followed by pairwise comparison with Dunnett's test (overall family error rate of 0.05) was executed with the controls being PLGA fibers and TCPS.

2.7 Schwann cell and primary neuron viability and alignment

Further bioassays with rat Schwann cells (RSC) and rat dorsal root ganglia (DRG) cells cultured on the fiber surfaces assessed the functionality of the electrospun fibers for peripheral nerve repair. As additional controls, two-dimensional polymer surfaces were prepared by dissolving PLGA/SAA blends in methylene chloride (50 mg/mL) and spin-coating onto glass coverslips (12 mm diameter, 0.15 mm thickness) at 2,000 rpm for 30 s using a spin-coater (Headway Research, Garland, TX). Before the spin-coating process, the coverslips were cleaned using Alconox (Alconox, New York, NY) and H₂SO₄:H₂O₂ (10:1 vol.%) solutions and stored in 70% ethanol. After spin-coating, residual methylene chloride solution was removed via vacuum in a Pyrex dessicator (Fisher, UK) for 24 h at room temperature. Prior to cell seeding, coverslips were placed in 24-well plates (Fisher) and sterilized under UV light at 254 nm for 900 s using a Spectrolinker XL-1500 UV cross-linker system (Spectronics Corp., Westbury, NY).

RSCs [RSC96, ATCC, Manassas, Va] were cultured (50,000 cells/well) in 24-well tissue culture plates in culture media consisting of Dulbecco's Modified Eagle's Medium (DMEM, Sigma-Aldrich, St. Louis, MO), 10% v/v fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA), 1% L-glutamate (Sigma) and 1% penicillin/streptomycin (Sigma). DRG cultures were prepared from P4 Sprague Dawley rats pups (Hilltop Laboratories Animals, Inc.) using methods adapted from Davies et al., 2004.⁵⁵ After dissection, DRGs were cultured (50,000 cells/well) in 24-well tissue culture plates with neurobasal medium (GIBCO BRL, Rockville, MD) supplemented with B27 (GIBCO BRL), 25 mM KCl, and penicillin/streptomycin (Invitrogen). DRGs and RSCs were cultured on the surfaces for 48 hours at 37 °C and 5 % CO₂, fixed in 4% paraformaldehyde, and labeled with an antibody against tubulin (Beta III isoform) and phalloidin respectively.

3. Results and discussion

3.1 Electrospinning parameters and procedures

Following synthesis and characterization, the SAA [Figure 1] and PLGA were blended and solubilized in an organic solvent mixture of dichloromethane (DCM) and dimethylformamide (DMF) [70:30]. In preliminary work, electrospinning the SAA polymers alone produced discontinuous fibers with frequent polymer beading. Blending the SAA with a higher molecular weight PLGA into a homogenous solution allows for the necessary polymer chain entanglement for continuous fiber fabrication and collection. Two compositions of the PLGA and SAA polymers (1:1 and 2:1, respectively) were blended and fabricated into fiber mats of both random and aligned orientation.

For the presented research, fibers were electrospun with a [70:30] solvent system of DCM and DMF respectively. Polymers in solvents with higher dielectric constants (DCM 8.93, DMF 38.25)⁵⁶ tend to have decreased fiber diameters because of reduced surface tension of the polymer jet at the needle tip.⁵⁷ The advantage of using a solvent system with DMF is the fabrication of reproducible fine fibers.

Although the applied voltage, needle gauge, and polymer solution flow rate remained constant, the distance from the charged needle to the grounded collector was influenced by the desired fiber orientation. For random orientation, the 30 cm X 30 cm square grounded target allowed for random collection of fibers from a distance of 10 cm. For aligned fiber orientation, the combination of the smaller rotating drum target and other metallic components in the collection set-up necessitated a smaller gap size of 5 cm for adequate fiber collection on the drum. Visual analysis of the fiber SEM images indicates that the surface morphology was not affected by the change in gap size (data not shown).

Several other methods are utilized for fabricating uniaxially aligned fibers: coverslips attached to rotating disks with double-sided tape,⁵⁸ grounded parallel bar electrodes,⁵⁹ high-speed rotating metal drum,⁶⁰ and rotating drill placed in front of a two dimensional grounded collector⁶¹). To the best of our knowledge, our set-up [Figure 2] is the first reported to use a Dremel tool to achieve fiber alignment. This system is attractive because of its simplicity and effectiveness in fabricating reproducible aligned nanofibers [Figure 3]. Electrospun fibers were consistently aligned with the Dremel tool set at ~5,000 rpm. Matthews et al. revealed that electrospun collagen fibers maintained random orientation at rotational speeds less than 500 rpm and significant fiber alignment along the axis of rotation when the speed of the mandrel was increased to 4,500 rpm.⁶²

3.2 Fiber characterization

The SEM micrographs demonstrate that all fibers were continuous and homogenous. Thermal analysis reveals one representative glass transition temperatures (T_g) for both the PLGA:SAA 1:1 and 2:1 blend (37°C and 36°C, respectively), indicating the absence of phase separation of the two blended polymers.

Fiber diameters were determined for each batch of electrospun fibers. Numerous fibers from several SEM micrographs were measured with ImagePro software. The pore size of the fiber mats was variable between samples and is directly related to the duration of fiber collection. A longer collection time resulted in a higher density of fibers, therefore, a decreased amount of porosity.

Previous research has demonstrated that axon extension and alignment is influenced by fibers with a wide range of diameters ranging from nanometers to hundreds of microns.^{2,12–15} As shown in Table 1, the fibers produced typically vary from 0.6 to 2.0 μm in diameter. Although the influence of fiber diameter on cell outgrowth was not specifically explored in this work, Wen et al. demonstrated that smaller diameter fibers influence the rate of neurite outgrowth; 5–30 μm diameter fibers produced faster neurite outgrowth than fibers 100 μm in diameter or larger.²

Fiber alignment, as shown in Figure 3, was quantified by manually tracing individual fibers in the SEM micrographs using NIH ImageJ software. Several micrographs were analyzed for both the random and aligned orientations for each polymer composition. Fiber orientation was normalized with respect to the horizontal axis and each angle was placed into a data bin of 10°. Angles had a range from -90° to 90° with 0° being designated as the parallel reference line. The orientation of the randomly distributed fiber mats is evenly distributed throughout all of the angle bins. Conversely, the oriented fiber mats have a distinct element of alignment with 68% and 75% of the fibers analyzed falling within $0 \pm 10^\circ$ for the PLGA/SAA [1:1] and PLGA/SAA [2:1] polymer compositions respectively.

Morphological characterization of the electrospun fiber mats reveal both randomly orientated and well-aligned fiber constructs with fiber diameters that are favorable for neurite and Schwann cell outgrowth and proliferation.

3.3 Drug release via hydrolytic degradation of the electrospun scaffolds

In both *in vivo* and *in vitro* conditions, the lower molecular weight poly(anhydride-ester) (SAA) is expected to degrade faster than the admixed PLGA.^{38,63–65} SEM was utilized to observe the mechanism of fiber degradation over 8 weeks of *in vitro* hydrolytic degradation. SEM micrographs of fiber mat samples at 0, 1, 3, and 8 week time points reveal predominantly intact fiber macro structures with increasing surface porosity visible on the PLGA/SAA fibers at 3 and 8 weeks (Figure 5). The fibers composed of PLGA alone had limited evidence of surface erosion. It is difficult to generalize the wide degree of clinical

peripheral nerve injuries; however, the rate of axonal elongation within a nerve conduit has been determined to be approximately 1 mm/day in a rat sciatic nerve injury model.⁶⁶ Following the formation of a fibrin matrix and Schwann cell bands on Bnger, axons typically begin to bridge a nerve gap two to three weeks after injury.⁶⁷ Therefore, it is necessary for a fiber scaffold within an NGC to maintain topographical features for several weeks following injury. These results are encouraging because the fibers are clearly intact at week 3 and begin to show signs of surface degradation at week 8.

Quantifying salicylic acid release via UV-spectroscopy and measuring the mass loss of the fiber mats demonstrate that the SAA polyanhydride completely degrades after 42 days (Figure 6). As hypothesized, fiber orientation does not significantly change the salicylic acid release profile from the scaffolds. Sustained bioactive release over 42 days is uncharacteristic of polymer systems with admixed therapeutics which typically release the active molecule in hours.⁶⁸ The release profiles of the 1:1 and 2:1 PLGA to SAA ratios demonstrate limited differences in cumulative percent salicylic acid release; however, the fibers composed of a 2:1 ratio of the polymers contain less salicylic acid. Two ratios of SAA to PLGA were chosen to demonstrate the feasibility of fabricating fibers with tailored amounts of intrinsic bioactive molecules, while maintaining surface morphology and topographical functionality.

3.4 Cell proliferation and morphology

Polymer scaffolds used for nerve regeneration must support cell viability and proliferation. Previous work has shown the encouraging biocompatibility of the two individual polymer components of the fibers (PLGA and SAA) when the polymer is presented in solution to L929 fibroblasts as a component of the cell culture media.^{69,70} This research demonstrates that the surface properties of PLGA/SAA polymer fiber mats are favorable for L929 fibroblast viability and sustained proliferation over 72 hrs. Williams et al. observed the sequence of sciatic nerve regeneration in a rat model, noting the significance of fibroblast cells.⁶⁶ The first week following conduit implantation involves the formation of a relatively acellular fibrin matrix bridging the nerve gap.⁶⁶ Within the second week, the fibrin matrix was infiltrated with Schwann cells, fibroblasts, endothelial cells, and perineurial cells. The proceeding weeks are followed by continued Schwann cell and fibroblast activity to lead axon migration, coordinate axon myelination, and form new blood vessels.⁶⁶ Additionally, fibroblasts and endothelial cells are abundant in the connective tissues which surround peripheral nerves, producing growth factors (i.e. NGF) known to be involved with axonal growth.⁷¹

In this live/dead assay, Calcein AM enters live cells and reacts with intracellular esterase to produce green fluorescence, and ethidium homodimer-1 only permeates the damaged membranes of dead cells to bind to nucleic acids and emit a red fluorescence. Figure 7A shows the increasing cell proliferation and stellate cell morphologies on PLGA/SAA [1:1] fiber, PLGA/SAA [2:1] fiber, PLGA fiber, and TCPS surfaces over 72 hrs.

As indicated by ANOVA followed by pairwise comparison with Dunnett's test, a significant difference exists between the PLGA fiber control and the PLGA/SAA [2:1] fibers, as well as between the TCPS control and the PLGA/SAA [2:1] fibers after 24 hrs of culture (overall family error rate = 0.05). No significant differences exist between either control and the experimental conditions for the 48 hr and 72 hr time points. Healthy cell proliferation is evident in all conditions with large regions of confluency after 72 hrs of cell culture, indicating a favorable tissue engineering substrate. Following validation of the fiber surfaces with the immortal fibroblast cell line, application relative Schwann cells and primary neurons were cultured on the fiber surfaces to confirm cell viability and topographical functionality.

3.5 Schwann cell and primary neuron viability and alignment

To further assess the fiber scaffolds ability to support and align relevant, proliferating cells, RSCs were cultured on fiber mats. Schwann cells are critical in the regeneration process because they proliferate to form longitudinal cell pathways (bands of Büngner) for guided axon growth.⁷² Aligned fiber scaffolds encourage the formation of linear Schwann cell processes that are the building blocks of the bands of Büngner. Although the random mats are not of particular interest as a functional nerve regeneration scaffold, RSCs were cultured on randomly orientated fibers to delineate the phenomenon of extended linear processes seen on the aligned substrates. As an additional control to evaluate Schwann cell compatibility on the surface of the chosen polymers, RSCs were cultured on two-dimensional spin-coated polymer surfaces. The RSC viability on the polymer two-dimensional surfaces did not statistically differ from the polymer fiber surfaces (data not shown). 84% and 63% of the RSCs had processes elongated in the direction of the fiber alignment ($0 \pm 10^\circ$) on the PLGA/SAA [1:1] and PLGA/SAA [2:1] fibers, respectively [Figure 8].

DRGs were additionally prepared from P4 rat pups and cultured on the various polymer fiber compositions and orientations to investigate the viability of a primary neuronal cell line and to confirm the topographical functionality of aligned fibers. β -tubulin staining revealed distinct neurite alignment along the oriented electrospun fibers [Figure 9A-C]. Differing from the fiber surfaces, the TCPS control surface was coated with poly-D-lysine to encourage DRG attachment and neurite extension. For the aligned fiber surface, DRGs extended neurites in the direction of the fiber alignment ($0 \pm 10^\circ$) 87% and 88% of the time on the PLGA/SAA [1:1] and PLGA/SAA [2:1] fibers, respectively. In comparison, Schnell et al. seeded whole chick embryonic (E10) DRG explants on aligned electrospun collagen/poly- ϵ -caprolactone nanofibers and similarly found 87.8% of the DRG extending neurites were oriented in the direction of the fibers after 24 hrs of culture.⁵⁹

4. Conclusions

These studies demonstrate that aligned salicylic acid-derived poly(anhydride-ester) electrospun fibers have significant potential as a platform for an internal lumen of synthetic nerve guidance conduits. Although injuries have a great deal of variation in the clinic, it is necessary for implanted biomaterials to maintain the majority of their structure several weeks following implantation to support Schwann cell bridging and axon re-growth across a gap. PLGA is an attractive base material for next generation nerve guidance conduits and their respective luminal fillers because of its mechanical strength and tailored degradation by altering the molecular weight, crystallinity, and/or proportions of glycolide to lactide monomers.⁷³ However, a drawback to these degradable polyester systems is the high and localized concentrations of two acidic organic molecules (glycolic acid and lactic acid) that often invoke an immunological response.^{74,75} The addition of a poly(anhydride-ester) that undergoes hydrolytic degradation to release salicylic acid will mitigate the inflammatory cascade.

The versatility of the described fiber system is shown by controlling the proportions of bioactive polyanhydride and PLGA, thereby achieving tailored concentrations of the released drug. Our *in vitro* findings suggest that an aligned internal fiber scaffold with a 1:1 or 2:1 ratio of PLGA to SAA is suitable for peripheral nerve regeneration applications. Recent studies by Wang et al. have shown that both neurites and Schwann cells extend longer processes on fibers with diameters in the large (1325 ± 383 nm) and intermediate (759 ± 179 nm) range when compared to smaller fibers (293 ± 65 nm).⁵⁸ Wang's findings are encouraging because our blended polymer fibers display diameters that are comparable to the fiber group with longest processes reported.

While the focus of this paper is on the fabrication and topographical functionality of bioactive polyanhydride fibers, the local release of a salicylate active anti-inflammatory agent at the site of NGC implantation has the potential to moderate the progression of inflammation and up-regulate cytokines involved in reorganization of the actin cytoskeleton during neurite outgrowth and regeneration.

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Figure 1. Chemical structure of salicylic acid-based polymer (SAA) and the hydrolytic degradation products: salicylic acid and adipic acid.

Figure 2.

(A) Schematic of electrospinning set-up for fabricating random fiber mats with grounded metal plate collector. Abutting 10 mL syringes with the back syringe filled with water and hydraulically pushed by an attached syringe on a syringe pump. The front syringe holds the polymer solution which is dispersed via a charged 18G blunt stainless steel needle. (B) Schematic of electrospinning set-up for fabricating aligned fiber mats with rotating mandrel collector. (C) Photo of electrospinning set-up with abutting syringes and rotating mandrel attached to a 300 Series Dremel. (D) Dremel tool fastened into a custom aluminum stand with a rotating mandrel for aligned fiber collection.

Figure 3. SEM images of electrospun fibers: (A) PLGA/SAA [1:1] random orientation, 1,490X (B) PLGA/SAA [2:1] random orientation, 1,540X (C) PLGA/SAA [1:1] aligned orientation, 1,500X (D) PLGA/SAA [2:1] aligned orientation, 645X.

Figure 4.

Histogram representing fiber orientation for PLGA/SAA [1:1] and PLGA/SAA [2:1] fibers collected on a two-dimensional grounded surface (random) and on a rotating mandrel (aligned). The percentage of fibers aligned at 0° (between -10° and 10°) [n=3 electrospun fiber mats for each condition] is PLGA/SAA [1:1] Aligned - 57 ± 15 ; PLGA/SAA [1:1] Random - 11 ± 1 ; PLGA/SAA [2:1] Aligned - 47 ± 2 ; PLGA/SAA [2:1] Random - 16 ± 3 .

Figure 5.

Sequential SEM images of randomly oriented fibers following *in vitro* degradation for the indicated time (0, 1, 3, and 8 wks). Surface erosion of the fibers is evident for the admixed conditions, but not visible for the pure PLGA samples.

Figure 6. Salicylic acid release from electrospun fiber mats for 42 days of *in vitro* degradation (n=3)

Figure 7.

(A) Live(green)/dead(red) images of L929 fibroblasts at 24, 48, and 72 hours of culture on PLGA/SAA [1:1] fibers, PLGA/SAA [2:1] fibers, PLGA fibers, and TCPS (B) L929 fibroblast viability as a percentage of all cells on culture surfaces [n=9]. Significant differences against the PLGA control are indicated by * and significant differences against the TCPS control are indicated by +. Significant differences are obtained by Dunnett's test using an overall family error rate of 0.05.

Figure 8.

RSCs cultured on PLGA/SAA [2:1] fibers with: (A) random orientation, (B) aligned orientation, (C) random orientation showing highlighted processes for length and orientation analysis. Scale bar 200 μ m in all images. (D) Histogram of RSC process orientation. A 0° angle indicates perfect cell alignment in the parallel direction of the fibers. (E) RSC morphology expressed as average RSC process length compared to a glass surface control.

Figure 9.

DRGs cultured on: (A) PLGA/SAA [1:1] fibers with random orientation, (B) PLGA/SAA [1:1] fibers with aligned orientation, (C) Poly-D-lysine coated TCPS. Scale bars are 200 μ m in all images. (D) Histogram representing DRG orientation (E) DRG morphology characterized as process length measured from the cell body on random (R) and aligned (A) fibers.

Table 1

Average fiber diameter (microns)

	Random	Aligned
PLGA/SAA [1:1]	$1.8 \pm 0.4 \mu\text{m}$	$2.1 \pm 0.7 \mu\text{m}$
PLGA/SAA [2:1]	$1.1 \pm 0.3 \mu\text{m}$	$0.6 \pm 0.2 \mu\text{m}$