Electrospun Poly(D,L-lactide) and Polyaniline Scaffold Characterization

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ABSTRACT: Neuromuscular disease or peripheral nerve damage can interrupt muscle contraction, but tissue engineered constructs can be created to combat this problem. Electrospinning provides a way to create a degradable nonwoven mesh that can be used to culture cells and tissues. Conductive polymers can be blended with other polymers to provide an electrical current to increase cell attachment, proliferation, and migration. We electrospun several polyaniline and poly(D,L-lactide) (PANi/PDLA) mixtures at different weight percents including the following PANi-PDLA solutions (w/v): 24% (83% PDLA/17% PANi), 24% (80% PDLA/20% PANi), 22% (75%PDLA/25% PANi), 29% (83% PDLA/17% PANi), and 29% (80% PDLA/20% PANi). Only the 75/25 electrospun scaffold was able to conduct a current of 5 mA. The calculated electrical conductivity for this scaffold was 0.0437 S/cm. Primary rat muscle cells were cultured on all three of the scaffolds and on tissue culture polystyrene as a positive control. Although the scaffolds degraded during this process, cells were still able to attach and proliferate on each of the different scaffolds. The cellular proliferation measurements showed no significant difference between the four groups measured. The conductivity and cellular behavior demonstrate the feasibility of fabricating a biocompatible, biodegradable, and electrically conductive PDLA/PANi scaffold. © 2009 Wiley Periodicals, Inc. J Appl Polym Sci 115: 1566–1572, 2010

Key words: polyesters; conducting polymers; biomaterials; scaffold; tissue engineering

INTRODUCTION

Neuromuscular junctions (NMJs) are specialized synaptic structures that occur at the motor neuron-skeletal muscle interface, connecting peripheral nerves to skeletal muscle, causing the muscle to contract.[1–4] After the motor neuron is depolarized, acetylcholine is released from the presynaptic membrane and diffuses across to the postsynapse muscle end-plate.[1,3–5] Once the threshold level is reached, depolarization of the muscle fibers leads to contraction.[1,3,4] Vehicular accidents, sports injuries, and shrapnel from military combat all lead to traumatic peripheral nerve (PN) damage.[6] Traumatic injuries account for more than 500,000 patients per year with 200,000 undergoing nerve repair procedures.[7,8] The contractile process can be interrupted by either injuries to the PN or a NMJ disease.[1,3,4,6,7,9] To repair the damaged nerve and muscle area many different cell types and exogenous factors are required.[6,7,9,10] Since the axonal repair process is either slow, 1 mm/day, or unable to occur, it is necessary to find a solution to help ameliorate this problem.[6]

Tissue engineering (TE) combines engineering with life sciences to provide a way to develop a construct that can be placed into multiple patients suffering from a NMJ disease or PN damage.[8,11–13] TE does this by creating, repairing, or replacing damaged tissues and organs by using a combination of cells, biomaterials, and tissue-inducing molecules such as growth factors.[13–15] The chosen scaffold material is important in determining the biocompatibility, degradation rate, and chemical and physical properties.[8,10,16,17] The material must also encourage cell migration, adhesion, and growth.[16,18] Electrospinning provides a way to create a nonwoven mesh with fiber diameters varying from tens of microns to tens of nanometers.[16,17,19,20] Numerous synthetic polymers have been electrospun, including polyvinyl alcohol, polyethylene-*co*-vinyl acetate, polylactic acid (PLA), polyethylene oxide, polyvinylchloride, polylactic-*co*-glycolic acid, and polyethylene glycol.[8,10,11,17–19,21] Electrospun scaffolds provide an extracellular-like matrix so that cells can attach before making their own extracellular matrix (ECM) and producing newly regenerated tissue.[17]

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TABLE I
Parameters Used for Each of the PDLA/PANi Solutions

Polymer (%, w/v)	PDLA (% polymer)	PANi (% polymer)	Distance (cm)	Rate (mL/hr)	Positive voltage (kV)	Negative voltage (kV)
22	75	25	20	1.00	13.0–13.5	5.0
24	80	20	25	1.00	17–18	2.0
24	83	17	25	1.00	14.5	2.0
29	80	20	22	2.00	17	2.0
29	83	17	25	2.00	11.0	2.0

Conductive polymers such as polypyrrole (PPy) and polyaniline (PANi) are of growing interest because of their unique conductive property that increases cell attachment, proliferation, migration, and differentiation.[8,11,21–24] For example, PC-12 cells differentiated into neural-like cells upon the addition of electrical stimulation and nerve growth factor on both PPy and PANi surfaces.[11,24] Li et al. showed that rat cardiac muscle cells were able to attach, migrate, and proliferate on PANi-gelatin electrospun fibers.[21] Since PANi has been shown to be biocompatible, it can be utilized in a scaffold to improve cellular functions.

The focus of this article is to combine PANi with poly(D,L-lactide) (PDLA) to create a conductive, biodegradable, and biocompatible scaffold for an eventual nerve-muscle construct.[11,15,22,25,26] Huang et al. first combined these two polymers to make a PLA-PANi-PLA block copolymer that was electroactive and supported C6 glioma cell attachment and proliferation.[22] Later, a PLA-PANi multiblock was synthesized to generate better mechanical properties.[11] The goal of this study is to electrospin a blend of these two polymers together to form a synthetic ECM with conductive properties to influence cell migration, growth, and attachment of muscle cells.[8,11,17,21–24]

METHODOLOGY

Electrospinning

Camphorsulfonic acid (CSA), PANi, and hexafluoro-2-isopropanol (HFIP) were purchased from Sigma-Aldrich (St. Louis, MO). PDLA was purchased from Lakeshore Biomaterials (Birmingham, AL). Several solutions were made for this study: 24% (83% PDLA/17% PANi) (w/v), 24% (80% PDLA/20% PANi) (w/v), 22% (75% PDLA/25% PANi) (w/v), 29% (83% PDLA/17% PANi) (w/v), and 29% (80% PDLA/20% PANi) (w/v). Each of the PANi-CSA-HFIP solutions were rotated on a Thermolyne specimix (Fisher Scientific, Pittsburg, PA). After 2 h, the PDLA was added and mixed for an additional 4 h. A syringe containing the polymer solution was placed into a syringe pump and an electric field was applied. The resulting fibers were collected onto an aluminum foil covered flat surface. The quantities of both PDLA and PANi, the extrusion rate, distance between the needle and plate, and both positive and negative voltages applied are listed in Table I. Scanning electron microscopy (SEM) was utilized to determine the fiber morphology and diameter for each of the copolymer solutions. A total of 22 fibers over four fields were analyzed for each of the PDLA/PANi groups. The solutions were then compared on repeatability, the amount of bead formation, the amount of spatter, and the amount of droplets deposited on the electrospun mat to optimize the total polymer weight percent (wt %).

Conductivity

After optimization of the total polymer weight percent, the electrospun mats were soaked in saline for ~ 5 min (to mimic a possible *in vivo* environment) before each was placed onto an electrode (Capital Advanced Technologies, Carol Stream, IL). A constant voltage of 20.00 V and a constant current of 1.545 A were applied using an E3646A dual output DC power supply (Agilent Technologies, Santa Clara, CA). The current output was measured by the E3646A power supply and reported in Table II. Electrical conductivity of each mat was also calculated using eq. (1), where l is the length of the mat, R is the electrical resistance, and A is the cross-sectional area.[27]

$$\sigma = l/(R * A) \quad (1)$$

Cell study

The optimized PDLA/PANi mats were then cultured with primary rat muscle cells to determine cell

TABLE II
Values for the Voltage Input, Current Input, Resulting Current Output, and Electrical Conductivity for 100% PDLA and the Three PDLA/PANi Electrospun Scaffolds

% Polymer (PDLA/PANi)	Voltage input (V)	Current input (A)	Current output (A)	Electrical conductivity (S/cm)
75/25	20.00	1.545	0.005	0.04371
80/20	20.00	1.545	0	0
83/17	20.00	1.545	0	0
100/0	20.00	1.545	0	0

toxicity, attachment, and proliferation. The soleus muscle was harvested from juvenile male Sprague-Dawley rats (Harlan, Dublin, VA) weighing ~ 125–150 g each. The cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Mediatech, Herndon, VA) with 10% fetal bovine serum Mediatech, Herndon, VA) and 1% penicillin/streptomycin under standard culture conditions (37°C, 5% CO_2). Mats were placed into a 48-well culture plate and ethanol was added to each well for 30 min. The mats were then exposed to UV light for 30 min on each side for sterilization. DMEM media was then added and the mats were soaked overnight before seeding at 20,000 cells/well (passage 4). The media was changed three times per week over the course of the 2-week period.

Cell toxicity and proliferation were measured using the CellTiter 96 AQueousOne Solution Cell Proliferation Assay (Promega, Madison, WI). The old media was removed and 200 μL of DMEM media plus 40 μL of the cell proliferation assay was then added to each well. The plates were incubated for 3 h and the absorbance measured at 490 nm using a SpectroxMax M2 spectrophotometer (Sunnyvale, CA). All the scaffolds were then fixed using a gluteraldehyde-methanol method and dried to preserve the cells. These scaffolds were then imaged using SEM to determine if cells had attached to each type of scaffold.

Differential scanning calorimetry

The miscibility of the 75/25 scaffold was determined by measuring the glass transition temperature of the blended polymer using differential scanning calorimetry (DSC). This was then compared with the glass transition temperatures of PDLA and PANi alone. A 22% PDLA solution was electrospun under the following conditions: 15 cm distance, 3.00 mL/h rate, a positive voltage of 12 kV, a negative voltage of 5 kV, and a mandrel rotational speed of 1788–1789 rpm. A 22% (w/v) PANi film was made by dissolving PANi in HFIP and then casting the polymer in a Petri plate. These were then placed into a TA Instruments DSC Q1000 (New Castle, DE) machine and measured in triplicate using a heat–cool–heat method.

Degradation study

An *in vitro* degradation study was carried out for 2 weeks on 22% PDLA and 22% 75/25 PDLA/PANi scaffolds. Scaffolds were cut into 1 × 4 cm pieces and weighed. Each sample was placed into a vial containing 10 mL of PBS. These were then placed in an agitated water bath at 37°C. Six samples of each group were removed at Days 7 and 14. The samples were vacuum dried and the weight measured. The weight loss percentage (*Wl*%) was calculated using eq. (2), where *Wi* is the initial weight and *Wf* is the final weight.[11,22,28]

$$Wl\% = 100 * (Wi - Wf)/Wi \quad (2)$$

RESULTS

PDLA/PANi scaffolds were electrospun for each of the following solutions (w/v): 24% 83/17, 24% 80/20, 22% 75/25, 29% 83/17, and 29% 80/20. Total polymer wt % was optimized for each of the three PDLA/PANi blends to (1) minimize the amount of polymer spattering, (2) minimize the amount of bead formation, (3) and electrospin with repeatability. SEM was utilized to ascertain fiber morphology and diameter for the three chosen solutions: 24% 83/17, 24% 80/20, and 22% 75/25 (SEM) (Fig. 1). All of the scaffolds have similar fiber morphologies and average fiber diameters, but the 75/25 scaffold fibers have a different range of diameters. Average fiber diameters for the three groups were as follows: (1) 1.18 ± 1.22 μm for the 83/17 scaffolds, (2) 1.19 ± 1.04 μm for the 80/20 scaffolds, and (3) 0.94 ± 0.65 μm for the 75/25 scaffolds. Fiber diameter ranges for each of the three solutions were: (1) 0.0291–5.48 μm for the 83/17 electrospun mats, (2) 0.0291–5.04 μm for the 80/20 electrospun mats, and (3) 0.1695–2.343 μm for the 75/25 electrospun mats. The 83/17 and 80/20 mats have almost identical fiber diameter ranges; however, the fiber diameter range for the 75/25 mat was smaller.

Scaffold conductivity was measured by subjecting nanofiber mats to 20 V at 1.545 A. Only the 75/25 solution displayed a current output of 5 mA at the maximum voltage and maximum current. The calculated electrical conductivity for this mat was 0.0437 S/cm. No output current was measured for the other two electrospun mats (Table II). This may be due to the constrictions of the device: maximum voltage of 20.00 V and a minimum measurable current of 0.001 A.

Scaffold biocompatibility and toxicity were measured using a cellular proliferation colorimetric assay. A negative control of the scaffold alone was also tested to determine if the scaffold would react with the proliferation assay. The averages for each of the scaffolds alone were 0.236 nm for the 83/17 scaffolds, 0.220 nm for the 80/20 scaffolds, and 0.221 nm for the 75/25 scaffolds. Therefore, the scaffolds did not react with the assay and it could be used to accurately measure proliferation. Rat primary muscle cellular proliferation was measured at Days 1, 3, 7, and 14 with cells alone on tissue culture polystyrene as a positive control and on each of the three

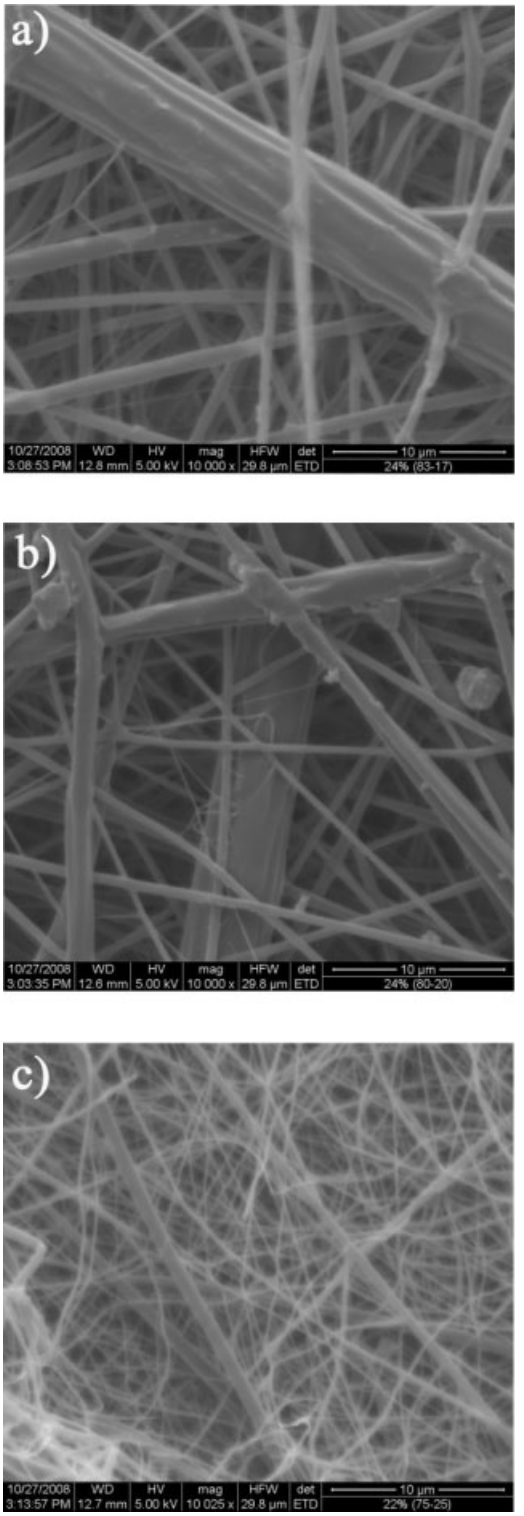

Figure 1 SEM micrographs of the electrospun solutions: (a) 24% (83/17) at 10,000×, (b) 24% (80/20) at 10,000×, and (c) 22% (75/25) at 10,025×.

mat types (Fig. 2). No significant differences were seen between the four different groups on each of the days measured. After SEM imaging, cells were found on all the different ratios of PDLA/PANi scaffolds for both Day 7 (data not shown) and 14 (Fig. 3). Multiple cell extensions were produced and attached to several fibers within each scaffold.

The miscibility of the 75/25 scaffolds was measured in triplicate using DSC. The results were then compared with measurements of PDLA and PANi alone. The average glass transition temperature (T_g) for the 22% 75/25 scaffold was 52 ± 0.4°C, the average T_g for the 22% PANi film was 96 ± 5.2°C, and the average T_g for the 22% PDLA mat was 52 ± 0.6°C. Since the T_g of our PDLA/PANi mixture was not in the middle of the single polymer scaffolds T_g, we conclude that the 75/25 polymer solution is a mixture instead of a blend.

A degradation study involving the 75/25 mixture and 22% PDLA scaffolds was conducted in an agitated water bath at 37°C. The *Wl*% of each sample ($n = 6$) was calculated and then averaged together for a final value. The 22% PDLA scaffolds showed very little degradation. At Days 7 and 14, the *Wl*% was 1.66 and 1.70, respectively. However, the 75/25 scaffolds displayed a larger amount of degradation. The *Wl*% for Day 7 was 15.15 and increased to 18.74 by Day 14.

DISCUSSION

Although PANi has been blended with several other polymers including electrospinning, we used a blend of PANi with PDLA in this study and successfully produced several different ratios of PDLA/PANi electrospun mats.[11,21,22,27] The amount of total polymer in solution (wt %) was very important to the electrospinning process. At low polymer weight percent lots of spattering occurred, but increasing the amount of polymer in solution decreased the spattering and allowed electrospun fibers to form.

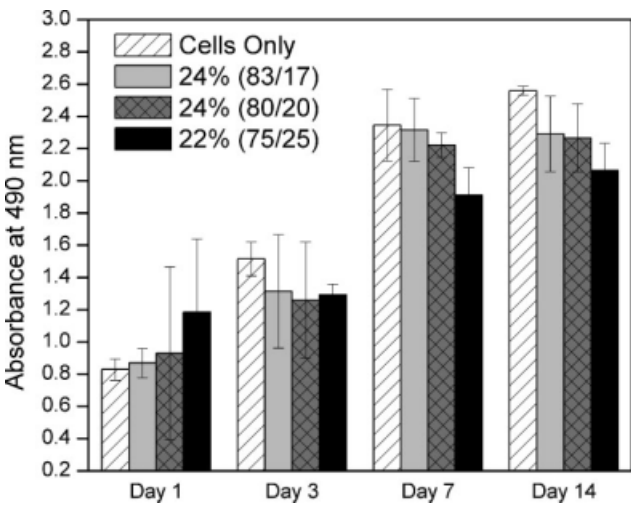

Figure 2 Rat muscle cell proliferation on the 24% 83/17 scaffolds, 24% 80/20, scaffolds, 22% 75/25 scaffolds, and the cells alone as a positive control.

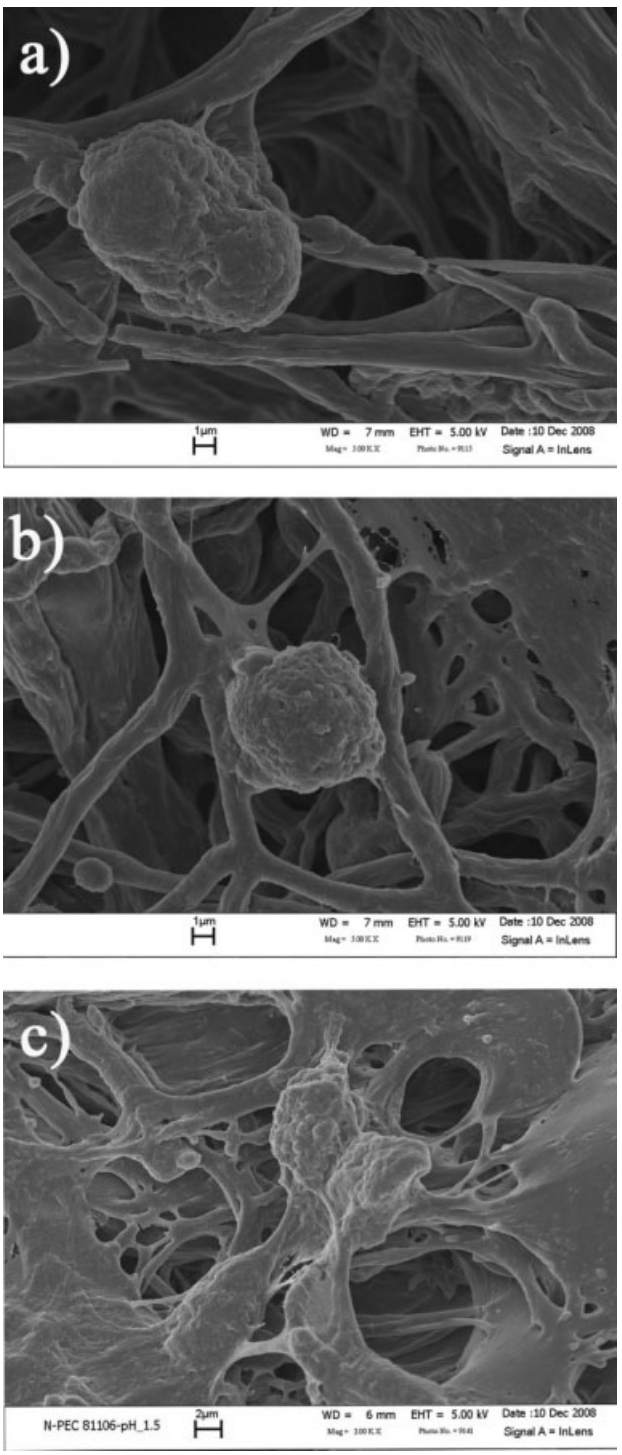

Figure 3 SEM micrographs at 5000× showing cellular attachment at Day 14 for the (a) 24% (83/17), (b) 24% (80/20), and (c) 22% (75/25) scaffolds.

Therefore, we chose our three solutions based on ease of electrospinning, repeatability, lowest amount of bead formation, and lowest amount of spattering.

SEM micrographs of the electrospun scaffolds were used to measure the average fiber diameter and range of 22 fibers for each of the three different solutions. Fiber diameter averages of the three were very similar: (1) 1.18 ± 1.22 μm for the 83/17 scaffolds, (2) 1.19 ± 1.04 μm for the 80/20 scaffolds, and (3) 0.94 ± 0.65 μm for the 75/25 scaffolds. Our fiber diameter averages are above what has been previously reported.[21,27,29] For example, Jeong et al. found that PLCL/PANi solutions of 85/15 and 70/30 had average fiber diameters of 423 ± 100 nm and 382 ± 102 nm.[27] The fiber diameter ranges for the 24% 83/17 (w/v) solution was 0.0291–5.48 μm, 0.0291–5.04 μm for the 24% 80/20 (w/v) solution, and 0.1695–2.343 μm for the 22% 75/25 (w/v) solution. PANi/PDLA fiber diameter ranges measured overlap both previously reported PANi fiber diameter ranges alone and when blended with other polymers.[20,21,27,29,30] For example, electrospun PANi alone fibers range from 300 to 1000 nm and PANi-PLCL fibers ranged from 100 to 700 nm.[27,29] However, our upper limit fiber diameter ranges are closer to the ranges of 500 nm to 5 μm for PANi with polymethyl methacrylate (PMMA) measured by Veluru et al.[30] It is possible that several smaller fibers fused together during the electrospinning process so larger fibers were formed; however, further analysis is needed to determine if this assumption is true.

All three electrospun PDLA/PANi polymer solutions were exposed to 20.00 V and 1.545 A, while the resulting current output was measured. Only the 22% 75/25 solution emitted a current: 5 mA resulting in a calculated electrical conductivity of 0.0437 S/cm. Several other articles have measured the electrical conductivity of CSA-doped PANi combined with other polymers. For example, Jeong et al. electrospun PANi-PLCL solutions at ratios of 0/100, 15/85, and 30/70 (%, v/v) and the electrical conductivity for each of these was 0.0015 S/cm, 0.0077 S/cm, and 0.0138 S/cm, respectively.[27] Veluru et al. electrospun PANi-PMMA mats which had an electrical conductivity of 0.00289 S/cm.[30] PANi-gelatin electrospun fibers in ratios of 15/85, 30/70, 45/55, and 60/40 yielded conductivities of 0.01 S/cm, 0.015 S/cm, 0.017 S/cm, and 0.021 S/cm.[21] Although our measured electrical conductance of 0.0437 S/cm was considerably higher than these three, it may be due to differences in calculations of w/v. We calculated our w/v solutions in g/mL, whereas the other three articles used either mg/mL or g/L.[21,27,30] However, our results fall on the electrospun PANi scaffold conductivity curve generated by Khan et al.[29] It is possible that our other scaffolds measured displayed conductivities that were too small to measure with our equipment. These studies may be repeated with a more sensitive conductivity meter.

A cellular assay was used to determine the toxicity and biocompatibility of the PANi-PDLA electrospun scaffolds. The cellular response to the assay was measured while the scaffolds were in their original well plates. Cellular attachment and

proliferation displayed no significant difference between any of the groups measured on any of the days in this study. Although the test results measure the activity of cells both in the well and on the scaffold, SEM imaging confirms the presence of muscle cells on the scaffolds. Therefore, the scaffolds are not cytotoxic (cells survived in the plates and on the scaffolds) and promote cell growth (based on the SEM pictures of cells with extensions). When the ethanol was added to each of the scaffolds, it caused the scaffolds to contract. The 83/17 scaffolds contracted to approximately 1/4 of the original size, the 80/20 contracted slightly less, and the 75/25 mats contracted the least but folded on top of themselves. After being removed from the ethanol, the scaffolds were very brittle, hard, and flat. Some of the scaffolds started to break apart and degrade by Days 7 and 14. The cell fixing procedure using gluteraldehyde and methanol lead to further scaffold degradation.

DSC was used to assess whether our conductive 75/25 solution was a true blend or a mixture. Three samples of each of the following groups were analyzed: (1) 75/25 scaffold, (2) 22% PDLA scaffold, and (3) 22% PANi film. The average T_g values for each were 52 ± 0.4°C, 52 ± 0.6°C, and 96 ± 5.2°C, respectively. From our results, we conclude that our 75/25 solution is a mixture rather than a blend as the T_g value was close to the T_g of PDLA alone.

A 2-week degradation study was performed to compare PDLA scaffolds to the conductive 75/25 PDLA/PANi scaffolds. Samples were removed from the PBS solution on Days 7 and 14 and vacuum dried. It was found that the PDLA dried samples curled and folded over on themselves. The 75/25 PDLA/PANi vacuum-dried samples displayed characteristics similar to the scaffolds in the cell study. They were hard, brittle, stiff, and in some cases, had broken into pieces. The *Wl*% for PDLA displayed very similar results for Days 7 and 14, 1.66 and 1.70, respectively. However, the 75/25 mixture had a higher degradation rate at Day 7, 15.15, and Day 14, 18.74. Our results are slightly higher than ones found by Huang et al. in a degradation study utilizing a poly(L-lactide)-PANI copolymer block in PBS.[22] This discrepancy could be due to the difference in the types of scaffolds and that our solutions contained a higher amount of PANi. Therefore, the addition of PANi to the solution does have an effect on the degradation rate.

CONCLUSIONS

PANi and PDLA were electrospun together to create a biodegradable, biocompatible, and electrically conductive scaffold. We were able to successfully electrospin nanofibers in various PDLA/PANi ratios. Three of these ratios were further analyzed: 24% 83/17, 24% 80/20, and 22% 75/25. Among these electrospun mixtures, only the 22% (75/25) scaffold conducted a significant current and had a calculated electrical conductivity of 0.0437 S/cm. Although the PDLA/PANi scaffolds degraded and shrunk, cellular data using primary rat muscle cells showed that all three of the scaffold types support cell adhesion and proliferation. Although the polymer degradation and shrinkage may prevent this polymer blend from being used as the primary component of a biomedical device, it may be used as a biocompatible coating on devices such as sensors. Future studies may focus on the use of other materials to blend with PANi to create a nerve-muscle construct.

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