Journal of Bioscience and Bioengineering
VOL. 112 No. 5, 501 – 507, 2011

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# Electrospun conducting polymer nanofibers and electrical stimulation of nerve stem cells

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Received 11 January 2011; accepted 9 July 2011
Available online 2 August 2011

**Tissue engineering of nerve grafts requires synergistic combination of scaffolds and techniques to promote and direct neurite outgrowth across the lesion for effective nerve regeneration. In this study, we fabricated a composite polymeric scaffold which is conductive in nature by electrospinning and further performed electrical stimulation of nerve stem cells seeded on the electrospun nanofibers. Poly-L-lactide (PLLA) was blended with polyaniline (PANi) at a ratio of 85:15 and electrospun to obtain PLLA/PANi nanofibers with fiber diameters of 195 ± 30 nm. The morphology, chemical and mechanical properties of the electrospun PLLA and PLLA/PANi scaffolds were carried out by scanning electron microscopy (SEM), X-ray photo electron spectroscopy (XPS) and tensile instrument. The electrospun PLLA/PANi fibers showed a conductance of $3\times10^{-9}$ S by two-point probe measurement. *In vitro* electrical stimulation of the nerve stem cells cultured on PLLA/PANi scaffolds applied with an electric field of 100 mV/mm for a period of 60 min resulted in extended neurite outgrowth compared to the cells grown on non-stimulated scaffolds. Our studies further strengthen the implication of electrical stimulation of nerve stem cells on conducting polymeric scaffolds towards neurite elongation that could be effective for nerve tissue regeneration.**



[**Key words:** Polyaniline; Conducting polymer; Poly-L-lactide; Neurite elongation; Nerve tissue engineering]

A successful tissue engineered product is such that the scaffold should have the ability to control the cellular response to direct the particular repair and regeneration of the concerned tissue. Nerve regeneration after injury is highly influenced by the cellular environment and several factors including the scaffold topography, stimulations (electrical, mechanical) or engineered channels affect the neurite outgrowth and axonal elongation (1,2). Directional cues can be provided using patterned nano extracellular matrix (ECM) structures, but they may not maintain the therapeutic levels of growth promoting signals which limits the nerve regeneration process *in vivo* (3). Growth factors such as the nerve growth factor (NGF) and brain derived neurotrophic factor (BDNF) have been delivered to the site of injury to promote axonal growth or methods such as the modification of biomaterial surfaces using peptides or proteins are being carried out. However, such methods involve limitations of retaining the bioactivity of the molecules and providing controlled and localized delivery to the site of injury (4–7). Electrical stimulation is an effective cue for stimulating cell proliferation and differentiations, mainly because bioelectricity plays an integral role in maintaining biological functions such as signaling of the nervous system. Moreover, recent studies on the electrical stimulation of cells cultured on conducting polymeric substrates have shown positive cellular influence in supporting and modulating certain kinds of tissue regeneration including the nerve (8,9).

Synthetic polymers have been widely studied for neural implantations, and bio-resorbable materials hold great flexibility in mechanical and chemical properties, lack antigenicity and avoid problems associated of chronic persistence (10). Polyglycolide (PGA), polylactide (PLA) and PLGA have been employed for use as nerve grafts, but the potential problem associated with PLGA is its fast degradation properties resulting in the collapse and inhibition of axonal growth (11). Poly(L-lactic acid) (PLLA) possesses good mechanical integrity, biodegradability and biocompatibility and it is utilized for the fabrication of scaffolds for nerve regeneration (11). PLLA has shown longer degradation behaviors and researchers have also used PLLA microfilaments as structural support for long lesion nerve gap regeneration (12). On the other hand, polyaniline (PANi) has attracted special attention because of its ease of synthesis, low cost, conductivity and environmental stability (13,14). Composite scaffolds of PANi with gelatin were fabricated by Li et al. (14) recently, where they found the fibers supporting the proliferation of H9c2 rat cardiac myoblasts (14). However, there are very limited studies carried out on the cellular response of bioengineered electroactive substrates for nerve tissue engineering. In this study, we investigated the effect of incorporation of PANi to PLLA towards the electro-spinnability of PLLA/PANi nanofibers,

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doi:10.1016/j.jbiosc.2011.07.010

evaluated the morphology, surface characteristics, mechanical strength and conductance of the electrospun nanofibers. Electrical stimulations of nerve stem cells cultured on conducting polymeric scaffolds were further carried out to enhance the neurite outgrowth, thus strengthening the application of electrical stimulus as a potential clue for nerve tissue regeneration, even without the presence of differentiation growth factors.

## MATERIALS AND METHODS

**Materials** PLLA with a molecular weight of 100,000 Da, was purchased from Polysciences (Warrington, PA, USA). 1,1,1,3,3,3-hexafluoro-2-propanol (HFP), polyaniniline emeraldine base (PANi-EB) of MW 65,000, camphorsulfonic acid (CSA) and hexamethyldisilazane (HMDS) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), horse serum (HS), antibiotics, and trypsin–ethylenediaminetetraacetic acid (EDTA) were purchased from Gibco (Invitrogen, Carlsbad, CA, USA). CellTiter 96 AQueous one solution was purchased from Promega, Madison, WI, USA.

**Electrospinning of nanofibrous scaffolds** PLLA was dissolved in HFP to obtain a 16% (w/v) solution and electrospun at a voltage of 10 kV (high voltage system, Gamma High Voltage Research, FL, USA), while the collector was placed at a distance of 15 cm from the needle tip to collect the nanofibers. Equal amounts of CSA and polyaniline-emeraldine base (PANi-EB) were dissolved in HFP, allowing the protonation of PANi-EB to a polyelectrolyte form. PANi-EB gets doped to polyaniline-emeraldine salt (PANi-ES) form, whereby the polymer undergoes an insulator to metal transition with change in conformation of the polymer backbone accommodating this electronic transformation (15). To the doped PANi solution, PLLA was added making a total concentration of 16% (w/v) and stirring was continued overnight. Before electrospinning, the polymer solution was filtered through a 0.22 μm filter unit and fed to a 5 ml standard syringe attached to 27 G blunted stainless steel needle using a syringe pump (KDS 100, KD Scientific, Holliston, MA, USA) at a flow rate of 1 ml/h. Electrospinning was carried out at a high voltage of 15 kV to obtain PLLA/PANi nanofibers. All experiments were performed in a humidity of less than 60% and a temperature of 22–24°C. Nanofibers were collected on 15 mm glass cover slips for cell culture experiments.

**Characterization of nanofibrous scaffolds** The morphology of electrospun nanofibers was studied using a scanning electron microscope (SEM) (JSM-5800LV, JEOL, Tokyo Japan) at an accelerating voltage of 10 kV, after sputter coating with gold (JEOL JFC-1200 Auto Fine Coater). The diameter of the electrospun nanofibers were also measured from the SEM micrographs using image analysis software (ImageJ, National Institutes of Health, Bethesda, MD, USA).

The wettability of the electrospun scaffolds was evaluated by water contact angle measurement using a VCA Optima Surface Analysis system (AST products, Billerica, MA). The droplet size was set at 0.5 μl. Average value of the contact angle was reported with standard deviation, after testing for a minimum of seven samples.

Attenuated total reflectance–Fourier transform infrared (ATR–FTIR) spectroscopic analysis of the electrospun nanofibrous scaffolds was performed on Avatar 380 spectrophotometer (Thermo Nicolet, Waltham, MA, USA) over a range of 400–4000 $cm^{-1}$ at a resolution of 2 $cm^{-1}$ with 64 scans per sample. The X-ray photoelectron spectroscopy (XPS) of PLLA and PLLA/PANi scaffolds were obtained from VG-Escalab 2201-XL Base system (Thermo VG Scientific, UK) at a take-off angle of 90°.

Tensile properties of the electrospun nanofibrous scaffolds were determined using a tabletop tensile tester (Instron 3345, Instron, Norwood, MA, USA) with a load cell capacity of 10 N. Rectangular specimens of dimensions 10 mm × 20 mm were used for testing at a crosshead speed of 10 mm/min at ambient conditions. The tensile stress and elongation at break were calculated from the obtained stress–strain curve.

The electrical conductivity of the electrospun scaffolds was determined using a two-point probe (Cascade TM, Microtech, Beaverton, OR, USA). At a voltage ranging from −40 to +40 V, the current versus voltage (I–V) curve of the nanofibers were obtained and the measurement was also further narrowed down to obtain a small signal I–V relation at a voltage range of −3 to +3 V to investigate the current response at low voltage.

Scaffold degradation studies are usually carried out in phosphate buffered saline (PBS), where the pH is near identical to the physiological conditions (16–18). Biodegradability of the electrospun PLLA and PLLA/PANi nanofibers were determined by incubating the scaffolds in a 24-well plate containing 1 ml of PBS of pH 7.4 at 37°C for 8 day period, with fresh additions of PBS every day.

***In vitro* nerve stem cells culture and electrical stimulation** Rat nerve stem cells (C17.2) were maintained in DMEM supplemented with 10% FBS, 5% HS and 1% penicillin/streptomycin/amphotericin-B (Invitrogen). Cells were maintained in a humidified $CO_2$ incubator at 37°C until confluency and fed with fresh medium every 3 days. Before seeding, cells were detached from the cell culture flask using trypsin-EDTA and viable cells were counted by trypan blue assay using a hemocytometer.

The electrospun nanofibrous scaffolds were sterilized under UV radiation, washed thrice with PBS and incubated with DMEM/F12 (1:1) media containing N2 supplement for 24 h before cell seeding. Cells were seeded at a density of 10,000 cells/$cm^2$ to study the cell proliferation on different electrospun scaffolds. Sterile tissue culture plates (TCP) for cell culture were purchased from Thermo Scientific Company (Singapore) and were used as such without any surface treatment (control).

The adhesion and proliferation of nerve stem cells on electrospun scaffolds were determined by colorimetric MTS assay (CellTiter 96 AQueous One Solution, Promega). After 2, 4, 6 and 8 days of cell culture, the cell-scaffold constructs were washed with PBS and incubated in DMEM containing 20% of MTS reagent for a period of 3 h. Thereafter, the aliquots were pipetted into the wells of a 96-well plate and placed into a spectrophotometric plate reader (FLUOstar OPTIMA; BMG Labtech, Offenburg, Germany), and the absorbance was read at 490 nm.

Electrical stimulation of NSCs (nerve stem cells) seeded on electrospun PLLA/PANi nanofibrous scaffolds was carried out using the procedure utilized by Ghasemi-Mobarekeh et al. (16). In short, cells were seeded on the electrospun nanofibrous scaffolds at a density of 10,000 cells/well and further incubated for 24 h for cell attachment and spreading. After 24 h, nerve stem cells were exposed to a steady potential of 1.5 V for a period of 60 min. To accomplish electrical stimulation, a silver electrode and a platinum electrode were inserted to opposite ends of the nanofibrous scaffold kept in culture medium and connected to a constant voltage (1.5 V). Taking into account the width of 15 mm PLLA/PANi scaffolds and constant voltage of 1.5 V, the electric field (voltage difference per unit distance) applied in this study is equivalent to 100 mV/mm.

**Cell morphology and neurite extension studies** Scanning electron microscope was used to observe the cell morphology after 4 days of cell culture on electrospun nanofibers. The samples were first fixed in 3% glutaraldehyde, rinsed with PBS (three times) and dehydrated with graded concentrations (50–100% v/v) of ethanol and finally added with HMDS. After air drying of HMDS, the samples were mounted onto a stub, coated with gold and observed under SEM.

After performing electrical stimulation, the cell-scaffold constructs were incubated for a period of 24 h and evaluated for their neurite outgrowth by SEM. The elongation of the neuritis were observed by SEM and the neurite lengths were measured from the SEM images using Image Analysis software, similar to those carried out by Schmidt et al. (19).

**Statistical analysis** Data presented are expressed as mean ± standard error of the mean and statistical analysis was performed using single factor analysis of variance. P value of $\leq 0.05$ was considered statistically significant.

## RESULTS AND DISCUSSION

Interaction between cells and polymeric scaffolds play a crucial role in determining the physiological behavior of cells such as cell adhesion, proliferation and differentiation (20,21). The use of polymeric substrates including conducting polymers to manipulate cell behaviors has recently gained interest mainly because electrical stimulus is important for cells involved in muscular and neural tissue regeneration (22). The interaction of cells to scaffolds depends on properties such as surface topography, hydrophilicity, electrical charges etc. (23). Electrospinning of pure PANi solution is rarely possible, though composite scaffolds with small concentration of PANi can be electrospun. A certain amount of polyaniline within the composite scaffold might be non-toxic to the cells and it could be electrically conductive enough to stimulate the cells and promote neurite outgrowth. We carried out *in vitro* experiments evaluating the capability of PANi containing composite (PLLA/PANi) nanofibers to modulate the differentiation phenotype of nerve stem cells for nerve tissue engineering.

**Morphology and chemical characterization of electrospun nanofibers** Electrospinning conditions were optimized during our study to obtain uniform randomly oriented bead free PLLA/PANi nanofibers by incorporating 15% of PANi with PLLA (PLLA:PANi 85:15). Fig. 1 shows the SEM images of electrospun PLLA and PLLA/PANi nanofibers produced by electrospinning. The fiber diameter of PLLA was 860 ± 110 nm and that of PLLA/PANi was 195 ± 30 nm. By incorporating PANi-CSA to PLLA, the net charge density of the solution might increase favoring the formation of fibers with low diameters. The presence of PANi-CSA can be considered similar to salt additions to electrospinning solutions, where it not only affects the viscosity but also the ionic conductivity and further the dielectric constant of the solution (24). Repulsion of electrostatic charges cause the stretching of fibers, thus reducing the fiber diameter of PLLA/PANi fibers. With inclusion of the same amounts of PANi to polycaprolactone/gelatin (PG) solution, Ghasemi-Mobarakeh et al.

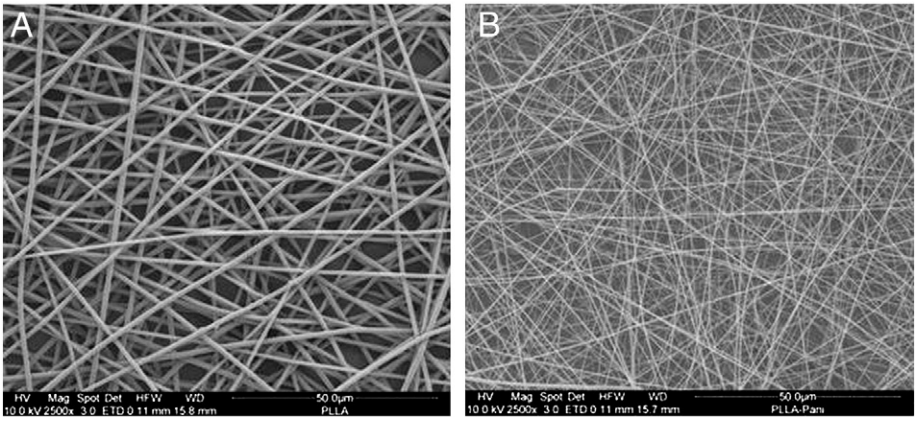


FIG. 1. SEM images of electrospun PLLA and PLLA/PANi nanofibers.

obtained composite PG/PANi (85:15) fibers with diameters of 112 ± 8 nm (16). Gelatin nanofibers with diameters of 803 ± 121 nm were produced by Li et al., and by incorporating higher amounts of PANi to gelatin (PANi/gelatin 40:60) they produced small diameter (61 ± 13 nm) fibers (14). It is also known that the size of the nanofibers regulate the adhesion, proliferation and differentiation of cells, besides its chemical properties (25,26). Incorporating higher amounts of PANi and CSA was found to produce beaded fibers instead of uniform randomly oriented fibers. A ratio of 85:15 of PLLA:PANi was therefore specifically chosen for the preparation of a conductive scaffold for nerve stem cell culture and for the electrical stimulation studies.

The surface hydrophobic–hydrophilicity of electrospun scaffolds has a major influence on cell adhesion and proliferation behavior. The hydrophilicity of a scaffold have an influence on the surface energy, which might influence the serum proteins to adhere on the scaffolds, and in turn govern the biological response, such as cell adhesion and proliferations (27). The wettability of PLLA/PANi nanofibers (126 ± 5°) was found similar to those of PLLA nanofibers (128 ± 7°). Since the surface hydrophobicity of both the scaffolds is comparable, the cell proliferations cannot be dependent on this factor alone. No significant differences in the contact angle values of PG and PG/PANi nanofibers were observed by Ghasemi-Mobarakeh et al. (16). Results of our studies shows that the incorporation of PANi has little or no effect towards the hydrophilicity of composite PLLA/PANi scaffold, compared to the PLLA scaffold. Similar results were also reported by Jeong et al., where they found that the incorporation of PANi into PLCL did not change the hydrophilicity/hydrophobicity of PLCL nanofibers (28). FTIR and XPS analyses were performed to evaluate the chemical composition of the electrospun scaffolds. The FTIR spectra (Fig. 2) of electrospun PLLA scaffolds showed peaks at 1760 $cm^{-1}$ and 1090 $cm^{-1}$ referred to the carbonyl and C–O stretch in PLLA, respectively. In addition to the carbonyl and C–O stretch related peaks, the electrospun PLLA/PANi scaffolds showed peaks at 1284 $cm^{-1}$ corresponding to the C–H in plane of deformation of PANi, 794 $cm^{-1}$ corresponding to the sulphonic acid groups and 1558 $cm^{-1}$ indicating that the aromatic ring is retained in the polymer (29,30). XPS analysis revealed the atomic ratios of C1s, N1s, O1s and S2p on the surfaces of the electrospun scaffolds and is shown in Table 1. The presence of S2p on PLLA/PANi scaffolds confirmed the presence of the dopant on the surface of the composite scaffolds by XPS analysis. The collective results from FTIR and XPS analyses suggest that the electrospun PLLA/PANi contain electrically conducting domains on the surface of the scaffolds, which make them effective to transfer the required electrical signals.

**Mechanical and electrical properties of electrospun nanofibers**

The combination of chemical, mechanical and electrical properties of the scaffolds plays an important role in tissue regeneration. Nerve grafts utilized for nerve tissue regeneration are applied as nerve guides, conduits or guidance channels. These involve the application of non-woven fabrics or electrospun nanofibrous sheets and hence we studied the mechanical properties of non-woven fabrics. Moreover, *in vivo* studies using electrospun fiber mats are also demonstrated to bridge large nerve gaps and direct axonal growth in peripheral nerve regeneration (31). The stress–strain curve for the electrospun nanofibers are shown in Fig. 3. The tensile strength and elongation

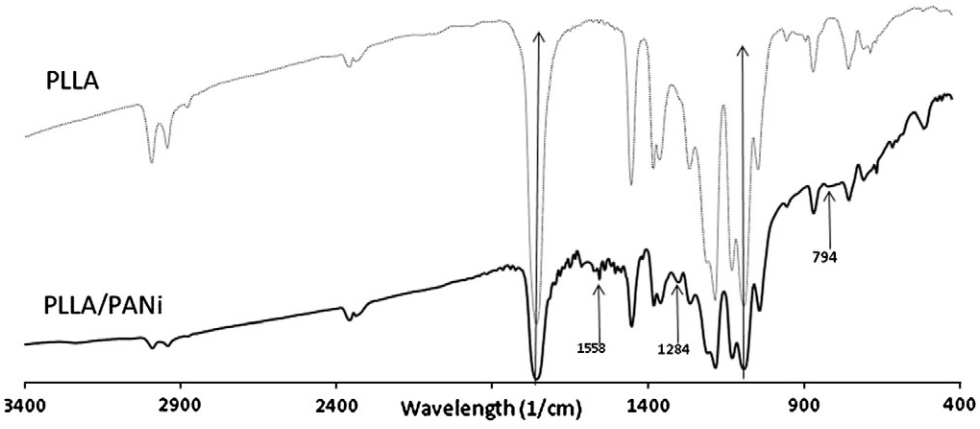


FIG. 2. FTIR spectra of electrospun PLLA and PLLA/PANi nanofibers.

**TABLE 1.** Atomic ratios of C1s, N1s, O1s and S2p on electrospun nanofibers measured by X-ray photoelectron spectroscopy.

| Scaffold | C atomic ratio | N atomic ratio | O atomic ratio | S atomic ratio |
|---|---|---|---|---|
| PLLA | 55.15 | - | 44.85 | - |
| PLLA/PANi | 55.63 | 1.13 | 42.29 | 0.95 |

at break were obtained as 4.69 MPa and 25%, respectively for PLLA nanofibers. PANi is brittle in nature and hence tailoring the mechanical properties of this polymer is essential while using the polymer for tissue engineering applications, despite its favorable electrical properties. The tensile strength of PLLA/PANi nanofibers decreased to 3.42 MPa, due to the presence of PANi in the composite scaffold, but with an increase in strain at break of 40%. An increase in the tensile strength of PG/PANi scaffolds were obtained by Ghasemi-Mobarakeh et al. compared to PG nanofibers, mainly because of the replacement of a weak natural polymer gelatin with a synthetic (strong) polymer PANi (16). Reports by Jeong et al. showed that the elongation at break for PLCL/PANi scaffolds (309.41%) decreased compared to the elongation at break for PLCL (391.54%) nanofibers (28). On the other hand, the tensile strength of fresh transected adult rat sciatic nerve is reported as $2.72 \pm 0.97$ MPa by Borschel et al. (32).

Various factors affect the conductivity of polymeric composites including the solvents used, doping agent, concentration of conductive polymer in the composite etc (33,34). We utilized CSA as the dopant and the conductance of electrospun PLLA/PANi fibers fabricated during this study was obtained as $3 \times 10^{-9}$ S. A linear I–V curve ideally represents the conductive property of a scaffold and during this study our PLLA/PANi (85:15) nanofibers showed a linear I–V curve, revealing its conductive properties (Fig. 4). It was therefore understood that the CSA dissolved in HFP protonated the emeraldine base (EB) of PANi producing the polyelectrolyte salt within the PLLA containing organic matrix. Our results are consistent to previous studies carried out by Li et al. (14) and Jeong et al. (28), where HFP was used for the fabrication of conductive gelatin/PANi and PLCL/PANi scaffolds, respectively. Moreover, the amount of PANi used in this study was sufficient to make the composite scaffold conductive and cause cell stimulation effects.

**Degradation of electrospun nanofibers** The degradation behavior of scaffold used for tissue engineering affect the concerned tissue of regeneration. We carried out the morphological evaluation of the electrospun scaffolds after soaking in PBS for a period of 8 days by SEM. Fig. 5 shows the change in morphology of PLLA and PLLA/PANi nanofibers after degradation. PANi being a non-biodegradable polymer, the inclusion of PANi in PLLA did not cause a significant or observable degradation in the morphology of the composite PLLA/PANi scaffold, by SEM analysis.

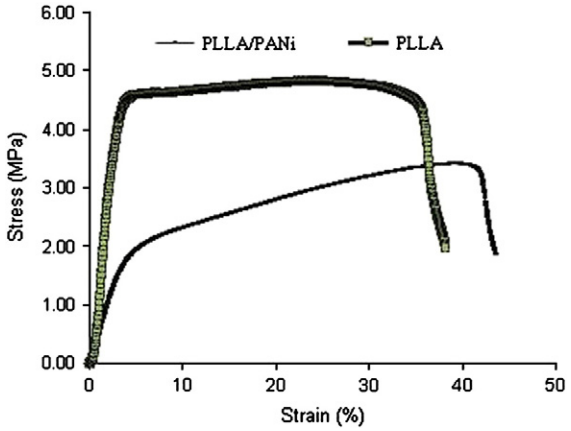


FIG. 3. Tensile graph of electrospun PLLA and PLLA/PANi nanofibers.

**Nerve stem cell proliferation and morphology on electrospun nanofibers** The nerve stem cell adhesion and proliferation on electrospun scaffolds were determined using the colorimetric MTS assay (CellTiter 96 AQueous One solution, Promega). The reduction of yellow tetrazolium salt in MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2(4-sulfophenyl)-2H-tetrazolium) to form purple formazan crystals by the dehydrogenase enzymes secreted by mitochondria of metabolically active cells forms the basis of this assay. The formazan dye shows absorbance at 490 nm and the amount of formazan crystals formed is directly proportional to the number of cells. Results of cell proliferation on the electrospun scaffolds are shown in Fig. 6. Fibers with small diameters are demonstrated to promote the spreading of nerve stem cells (C17.2 cells) than the fibers with large diameters (34). He et al. demonstrated that small diameter fibers facilitate the adsorption of proteins and other nutrients from the culture media due to their high surface area-to-volume ratio and the cells might require little energy to migrate and spread over small diameter fibers than the large diameter fibers (35,36). Proliferation of nerve stem cells on PLLA/PANi nanofibers showed that the PANi containing scaffolds are non-toxic and the proliferation of nerve cells on PLLA/PANi scaffolds ($195 \pm 30$ nm) were higher than the cell proliferation on PLLA nanofibers ($860 \pm 110$ nm) after 8 days of cell culture. However, higher amounts of PANi within the composite can be a hindrance to cell proliferations and optimized concentration of PANi within the composite and electrospinnability to obtain bead-free fibers are achieved during this study. Studies by Li et al. showed similar results, where they studied the proliferation of myoblasts on gelatin/PANi nanofibers with different concentrations of PANi (14). Results of their study showed that the proliferation of H9c2 cells was higher on both 15% and 30% PANi containing gelatin nanofibers compared to both TCP and gelatin scaffolds, after 6 days of cell culture.

Morphological observation of differentiated nerve stem cells grown on TCP, electrospun PLLA and PLLA/PANi nanofibers were carried out by SEM and are shown in Fig. 7. The nerve stem cells appeared spindle shaped on TCP, and the cell morphology on PLLA/PANi nanofibers was better than the morphology of cells attached on PLLA fibers (lost neurite extension and appeared flat). Cells on PLLA nanofibers were less spread and did not show its typical phenotype. Moreover, the neurite extension of cells on electrospun PLLA/PANi nanofibers was more discernible than the neurite extension on PLLA scaffolds. The phenotype of the differentiated nerve stem cells (C17.2 cells) was retained for cells grown on PLLA/PANi scaffolds, which indicates that the incorporation of PANi within the composite scaffolds did not affect NSC differentiations.

Neurite extension is an important phenomenon of nerve cell migration, which is dependent on the chemical, mechanical and electrical properties of the substrates (37,38). Electrospun aligned fibers provide topographical cues to direct and enhance the neurite outgrowth than the random fibers (39). The unidirectional contours of aligned fibers are demonstrated to provide physical guidance to the cells, facilitating the axon path-finding and accelerating the nerve tissue regeneration (40). Both the topographical cues and electrical stimulations were studied together by Xie et al. and they found that the effect of electrical stimulation on random fibers were higher than the effect on uniaxially aligned fibers, based on neurite length measurements (41). Scaffold that can act as a temporary substrate to stimulate cells of nerve tissue regeneration via electrical stimulation is fabricated in this study. Electrical stimulation of cells on electrospun PLLA/PANi scaffolds were carried out for a period of 60 min at a constant electric field of 100 mV/mm, after 24 h of cell seeding time (16). Control experiments were also carried out by seeding cells on PLLA/PANi nanofibers, but without electrical stimulation. The *in vitro* application of electrophysiologically relevant DC fields from 1 to 10 V/cm has been described to affect cell migration, alignment of nerve cells, increased collagen production and even

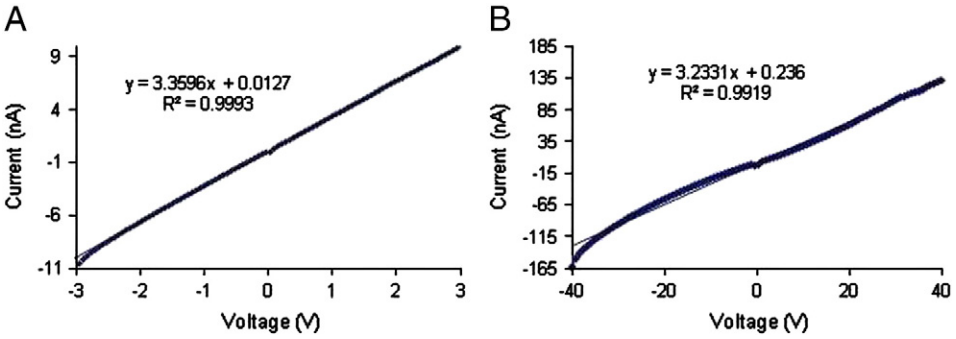


FIG. 4. I–V curve at a voltage range from (A) −3 V to +3 V (B) −40 V to +40 V for the electrospun PLLA/PANi nanofibers.

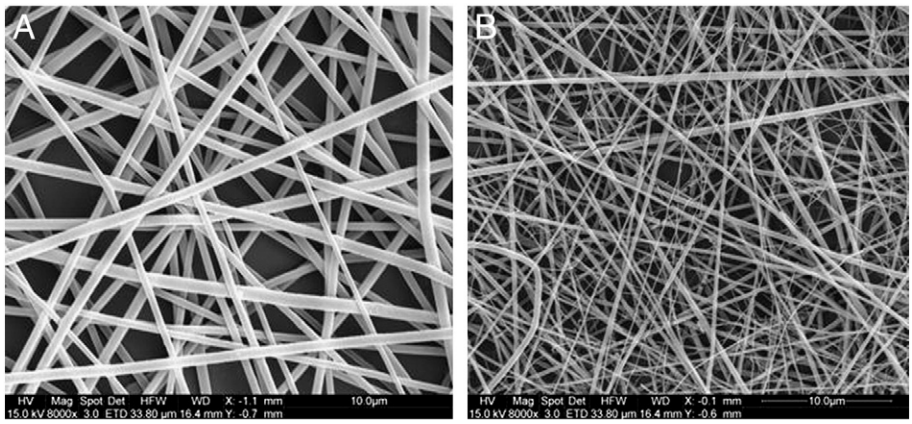


FIG. 5. SEM images of degradation of (A) PLLA (B) PLLA/PANi nanofibers after 8 day period.

differentiation of embryonic stem cells by various researchers (42,43). Significant increase in the outgrowth of neuritis was observed for cells electrically stimulated after culturing on PLLA/PANi nanofibers. Figs. 8A and B show the differences in the elongation of neuritis for cells cultured on PLLA/PANi nanofibers, with and without electrical stimulation. Electrical stimulation across a biomaterial might alter the local electrical field of ECM proteins and possibly modify the protein adsorption to polymeric surfaces and subsequently affect the neurite outgrowth (44). Cells on the electrically stimulated PLLA/PANi nanofibers showed a definite extension of neurite length, which was also higher than the neurite outgrowth observed for cells grown on PLLA nanofibers. The length of the neurite outgrowth was quantified using Image analysis software, and the average neurite length of cells cultured on PLLA/PANi scaffolds after electrical stimulation was found to be $24 \pm 4\,\mu m$, compared to $15 \pm 3\,\mu m$ without electrical stimulation. The neurite extension of nerve cells on electrically stimulated PCL/Gelatin/PANi nanofibers with conductance of $0.02 \times 10^{-6}$ S was reported as $30 \pm 1\,\mu m$ (16). We observed that the conductance of PLLA/PANi scaffolds was lesser than that fabricated by Ghasemi-Mobarakeh et al. (16) for PCL/Gelatin/PANi scaffolds ($0.02 \times 10^{-6}$), where they found the neurite extension of as much as 30 μm after electrical stimulation (16). Longer neurite length ($30 \pm 1\,\mu m$) was observed in our previous study due to the higher conductivity of PCL/Gelatin/PANi scaffolds, compared to PLLA/PANi scaffolds fabricated in this study. Moreover, the presence of gelatin which is a natural polymer in the structure of conductive scaffold of our previous study also contributed to an increase in the neurite length to a greater extent compared to the neurite length obtained for scaffolds of the current study. However, a comparatively low conductance PLLA/PANi scaffolds was able to produce a significant improvement in neurite outgrowth upon electrical stimulation ($15 \pm 3\,\mu m$ to $24 \pm 4\,\mu m$). On the other hand, even with the presence of gelatin and higher conductivity of PCL/Gelatin/PANi scaffold of our previous study, the difference in neurite length of the current study is not lower tremendously than those on PCL/Gelatin/PANi (meaning the maximum neurite length obtained for PCL/Gel/PANi was 31 μm and those for PLLA/PANi was 28 μm). Hence it can be concluded that low conductance fibers can cause neurite extension by using suitable scaffold compositions and more investigations are required in this direction to maximize the effect of conductance, scaffold compositions and electrical stimulation.

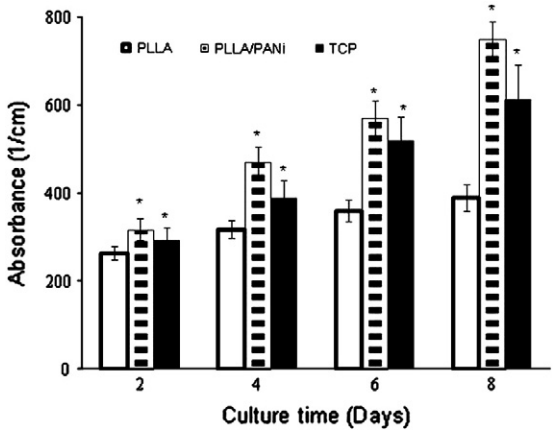


FIG. 6. Nerve stem cell proliferation on TCP, electrospun PLLA and PLLA/PANi scaffolds by MTS assay.

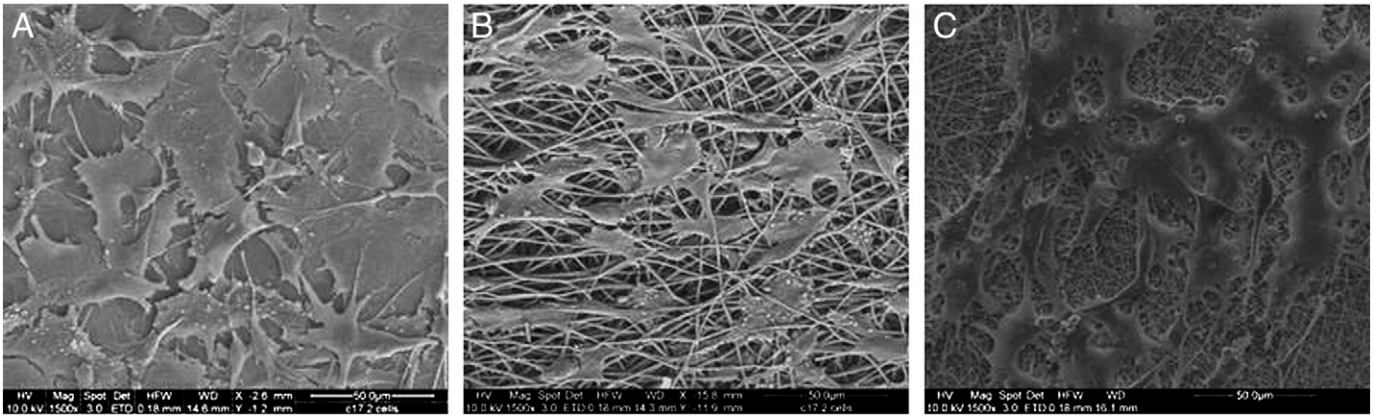


FIG. 7. SEM images of nerve stem cells on (A) TCP, (B) electrospun PLLA, and (C) PLLA/PANi nanofibers after 4 days of cell culture.

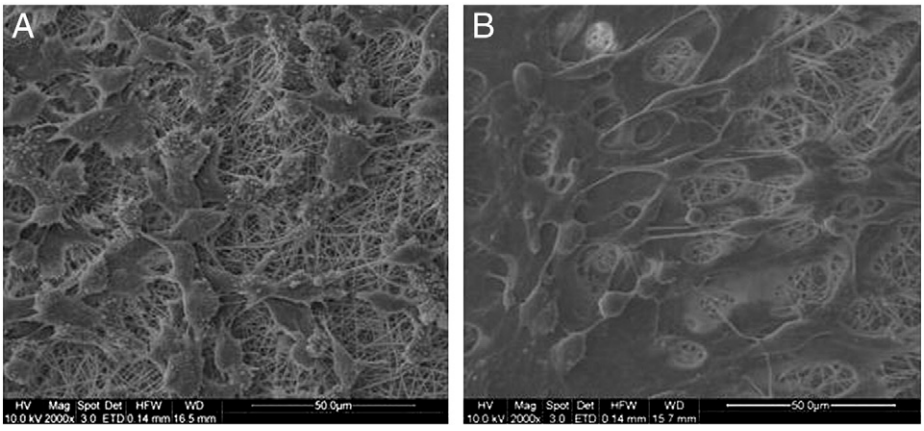


FIG. 8. SEM images of nerve stem cells on PLLA/PANi nanofibers. (A) Non-stimulated. (B) Electrically stimulated for 60 min period.

Our results show that the electrical stimulation of nerve stem cells on conducting polymeric scaffolds of PLLA/PANi can stimulate the differentiation or neurite elongation, highlighting the relevant application of electrical impulses as target signals for nerve tissue regeneration. Myotube formation, which is an important attribute in restoring muscular functions, was also found enhanced after growing cells on fibers (PLCL/PANi) with electrically conductive properties by Jun et al. (45). Results of our study might further encourage the application of stimuli responsive materials and introduction of various physical or electromechanical/mechanistic approaches to promote desirable cellular behavior and accelerate tissue regeneration.

Tissue engineered construct seeded with nerve stem cells and electrically stimulated, allowed for the dominant outgrowth of neurite essential for peripheral nerve regeneration. Electrospun PLLA/PANi scaffolds fabricated were found to possess the nanoscale features of native ECM, had the mechanical and electrical properties suitable for nerve tissue engineering. *In vitro* nerve stem cell culture on composite conductive scaffolds demonstrated cell biocompatibility of electrospun conductive PLLA/PANi scaffolds similar to those observed on biodegradable PLLA scaffolds. Moreover the electrical stimulation of nerve stem cells on PLLA/PANi scaffolds showed higher neurite extensions, which facilitate the regeneration of nerve. Results of this study encourage further research in the field of electromagnetic stimulations of nerve cells and mesenchymal stem cells towards neuronal cells for accelerated nerve regeneration. Our study emphasizes the need for more detailed research on the mechanisms behind the influence of mechanical and electrical properties of scaffolds in regenerating physiologically robust nerve tissue.

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