

# Electrospun Nanocomposite Nerve Guidance Conduits for Peripheral Neural Regeneration

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## ABSTRACT

The nervous system is prone to degeneration and very difficult to repair. Neural tissue engineering may provide a promising strategy for restoration of damaged nerves. In the current study, a highly aligned nanostructured neural scaffold was fabricated via electrospinning and electrospraying techniques. In particular, core-shell bovine serum albumin (BSA) loaded poly (D, L-lactide-co-glycolide) (PLGA) nanospheres were electrosprayed into electrospun poly- $\epsilon$ -caprolactone (PCL) microfibers for the first time. Our results showed that the introduction of BSA-loaded PLGA nanospheres can alter the scaffold's surface properties, such as increased nano surface roughness and wettability while the aligned microfibers can further guide axon propagation. More importantly, the PLGA nanospheres can promote neural cell adhesion and proliferation and sustainably release bioactive factors for further enhanced neural tissue regeneration.

**Keywords:** nanocomposite, nerve, electrospinning, electrospraying, nanosphere

## 1 INTRODUCTION

Chronic and acute peripheral nerve damage presents a preponderance of clinical problems all over the world [1]. At present, the autograft is considered as a gold standard for neural regeneration. However, this method replaces damaged tissues using healthy ones and results in complication and dysfunction in donor sites. Given the limited regenerative capacity of the neural system and ineffective clinical therapeutics, the development of novel strategies to improve and guide neural tissue regeneration is highly desirable. Neural tissue engineering approaches where biomaterials, biologics, and viable cells are integrated together provides an attractive option for improving the therapeutic effects when compared to traditional clinical approaches.

Numerous scaffolds manufacturing approaches and materials have been introduced into neural tissue engineering. Among them, electrospinning is one of the most popular scaffold fabrication approaches [2]. This technique can create 3D fibrous scaffolds with high surface area and porosity which is similar to the structure and

dimension of human tissues' extracellular matrix (ECM) [3]. In addition, it is easy to control the electrospinning process to get desirable topography (aligned or random orientation). Many studies have revealed aligned electrospun neural scaffolds can promote neural cell growth and guide neurites both *in vivo* and *in vitro* [4]. In the view of materials, there are a vast majority of polymers have been used for electrospinning. Synthetic polymers, such as PCL and PLGA, were extensively investigated for neural tissue engineering applications due to their excellent biocompatibility, biodegradability, and ease of functionalization [5].

Although there are increasing developed scaffolds for neural regeneration, the ideal neuroprosthetic devices and nerve guidance conduits are still absent. Ideally, a nerve construct should meet the following criteria: i) excellent biocompatibility; ii) suitable mechanical properties and conductivity; and iii) biomimetic morphological and chemical features for axonal guidance. From the biomimetic point of view, here we report a novel nanocomposite fabricated by electrospinning and electrospraying techniques for improved neural regeneration. For this purpose, PCL was electrospun into highly aligned fibers in order to guide neurite extension to the target injured sites. Furthermore, core-shell PLGA nanospheres with BSA were fabricated using coaxial electrospraying technique to provide sustained nutrition for our neural scaffolds.

## 2 EXPERIMENTAL DETAILS

### 2.1 Fabrication of nanocomposites

PCL was dissolved in chloroform at a concentration of 12 % (w/v) under sonicating for 1 h to form a clear solution. The solution was fed into 5 mL syringe attached to a 26 G steel needle and electrospun at a flow rate of 4.5 mL/h with an applied voltage of 5 kV. The electrospun fibers were collected on rotating mandrel to form aligned structure. As control group, random fibers were obtained by collecting on static foil.

BSA loaded core-shell PLGA nanospheres were fabricated by electrospraying and deposited on the surface of electrospun fibers. Briefly, a core-shell needle with 20 G outer and 26 G inner was fed with 1 mg/mL BSA aqueous

solution as core and 2.5% (w/v) PLGA solution (dissolved in acetone) as shell. 7 kV voltage was applied to form BSA embedded core-shell nanospheres. As control, equivalent bare BSA was directly sprayed onto PCL scaffolds in the absence of PLGA nanospheres. All samples were frozen overnight followed by lyophilizing to remove remain solvent.

In addition, PLGA microspheres with BSA were prepared using via water/oil/water double emulsion solvent extraction technique to compare the release behavior with core-shell nanospheres *in vitro*. The method of microspheres fabrication just followed an established protocol [6].

## 2.2 Characterization of nanocomposites

Scanning electron microscope (SEM, Zeiss NVision 40 FIB) was employed to examine the surface morphology of electrospun nanocomposites. Surface wettability properties of the electrospun nanocomposite were measured by a contact angle analyzer (DSA4, Krüss) equipped with a camera. A tabletop axial tester was employed to measure the tensile mechanical properties under a load cell capacity of 50 N. The scaffolds were stretched as a rectangular strips of 10 mm × 20 mm dimensions. Young's modulus was calculated from obtained tensile stress-strain curve of each scaffold in the linear region. For the drug release study of electrosprayed nanospheres, 10 mg spheres placed in microcentrifuge tubes containing 1 mL of phosphate buffered solution (PBS) at 37 °C in an incubator. At the end of each immersion period, the samples were centrifuged and supernatants were analyzed by BCA™ Protein Assay Reagent kit (Pierce Biotechnology) and the BSA content was quantified. Each sample was prepared in quintuple.

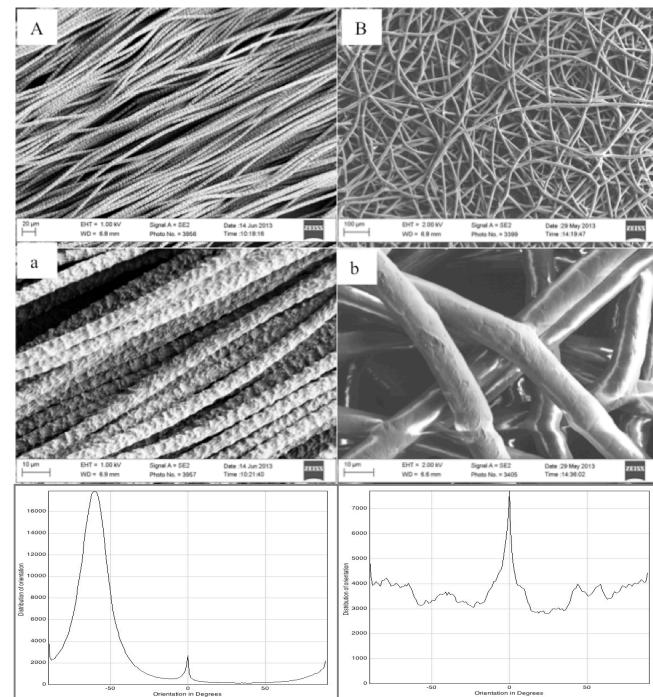
## 2.3 PC-12 cell proliferation and immunocytochemistry studies

PC-12 cells were cultured in RPMI 1640 (ATCC), a high glucose media, supplemented with 10% horse serum (ATCC) and 5% fetal bovine serum (ATCC) and 1% L-glutamine (Sigma-Aldrich) and penicillin-streptomycin (10,000 units/mL Penicillin, 10,000 µg/mL Streptomycin) (Gibco). For the proliferation study, scaffolds were cut into 5 mm diameter circles and PC-12 cells were seeded at a density of 30,000 per scaffold. A CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay (MTS assay, Promega) was used to quantify the cell number at day 2, 4, and 6. Cell differentiation and extension were visualized by Laser Scanning Confocal microscopy (LSCM 710, Zeiss). Specifically, PC-12 cells were cultured in nerve growth factor (NGF) containing media to induce differentiation for 10 days. The differentiated PC-12 cells were stained by mouse anti-Tuj1 (1:1,000, Covance) conjugated Alexa Fluor 594 goat anti-mouse (Life technologies) for cell body and 6-diamidino-2-phenylindole (DAPI) (Life technologies) for nuclei.

All quantitative data were expressed as means ± standard error mean and analyzed with student t-test to make pair-wise comparisons. Statistical significance was considered at p<0.05.

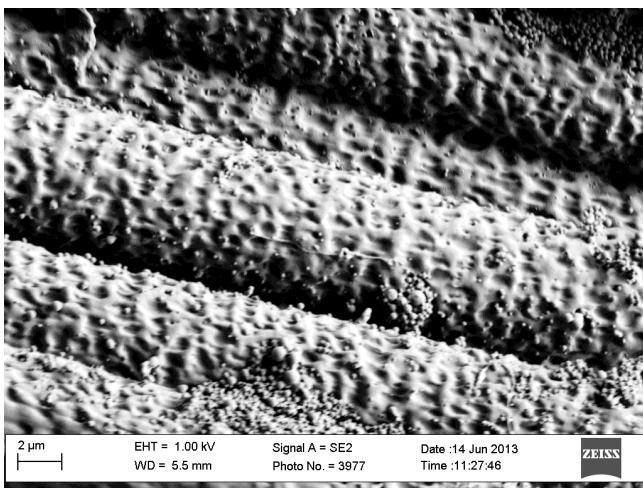
## 3 RESULTS AND DISCUSSION

SEM micrographs illustrate the electrospun aligned (Figures 1A and 1a) and random microfibers (Figures 1B and 1b) can be obtained via a rotating mandrel and a static foil. Analysis of orientation indicates much uniformer angle distribution in the aligned fiber mat than random fiber mat. It is known the topography of scaffolds influences cell behaviors, such as cell adhesion, proliferation, and differentiation [7]. In neural regeneration, the highly aligned topographical cue can guide neural cell outgrowth and neurite extension toward the synaptic targets which is important for rebuilding complex neural network [8].

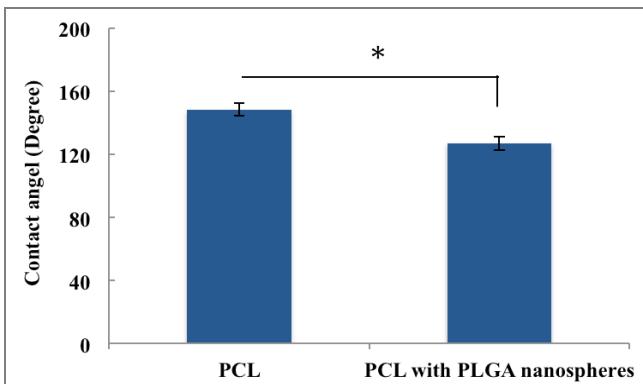


**Figure 1.** SEM images of electrospun microfibrous scaffolds at high and low magnifications: (A) and (a) highly aligned PCL scaffolds; and (B) and (b) random PCL scaffolds. The bottom images are fiber orientation analysis via OrientationJ plug-in for imageJ.

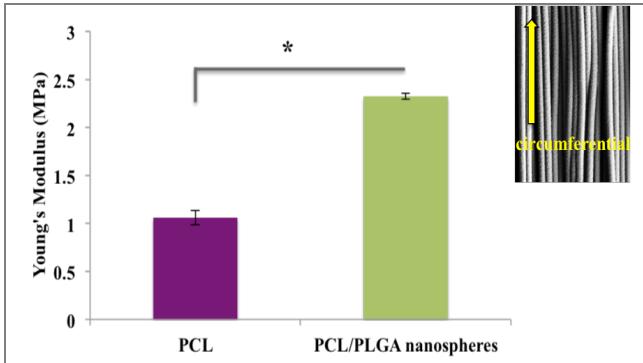
Figure 2 reveals that the aligned electrospun nanocomposite has porous surface structure and BSA loaded nanospheres were uniformly distributed on the surface. The nanospheres may render scaffolds with improved nanoroughness. The improved nanoroughness is estimated to increase neural cell growth in comparison with smooth scaffolds [9].



**Figure 2.** SEM images of highly aligned nanocomposite scaffolds: PCL scaffold with core-shell PLGA nanospheres



**Figure 3.** Reduced contact angle after electrospraying nanospheres. n=5, \*p<0.05.

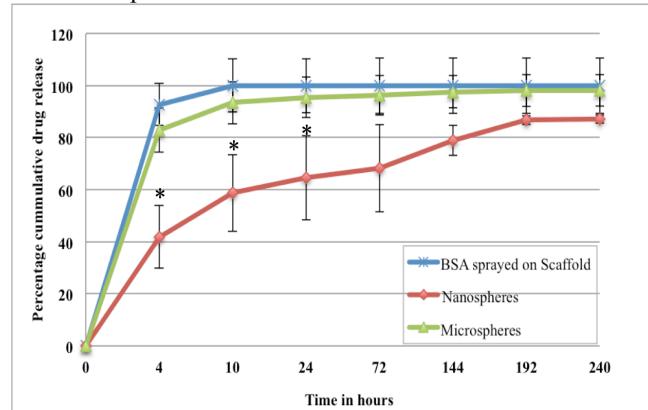


**Figure 4.** Greatly increased mechanical property of nanocomposite scaffolds. n=5, \*p<0.05 when compared with control groups.

Figure 3 shows the decreased contact angle was obtained after involvement of PLGA nanospheres onto the PCL aligned fiber mat, which means a more hydrophilic surface was created. It has been shown that hydrophilicity can be related to increased specific protein adsorption on

scaffolds surface and can contribute to enhanced cell response [10]. Furthermore, the nanospheres increased Young's modulus of scaffolds (Figure 4) in the circumferential direction (scaffolds are applied in this direction in most cases) from  $1.06 \pm 0.07$  MPa to  $2.33 \pm 0.03$  MPa. Enhanced mechanical properties are beneficial to both implantation operation and withstanding repeating loads *in vivo*.

Neurotropy is a key element in neural regeneration. Neurotrophic factors can influence neural cell development, survival, outgrowth, and branching on the level from molecular interactions to macroscopic tissue responses [11]. Currently, neurotrophic factors are facing ongoing delivery issues, such as short-term retention, quick half-life in circulation, and quick loss of biological activity *in vivo* [12]. Here we evaluated the BSA release profile of our nanospheres and compared with microspheres drug delivery device. Cumulative release results (Figure 5) demonstrate that PLGA nanospheres can release BSA for a longer time with much lower initial burst release than microspheres and bare BSA. More than 90% of BSA was released from the microspheres and BSA sprayed scaffolds at the initial 10 h. In contrast, there is less than 60% BSA released from core-shell nanospheres at the same time.

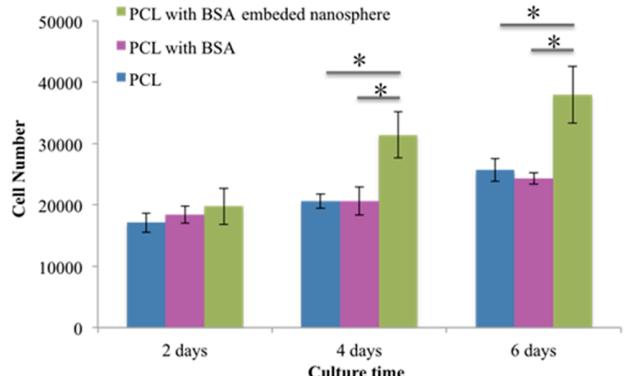


**Figure 5.** Cumulative BSA release profiles of nanosphere, microsphere and bare BSA in scaffolds, Data are mean  $\pm$  standard error mean, n=5, \*p<0.05 when compared other groups at 4, 10, and 24 hours.

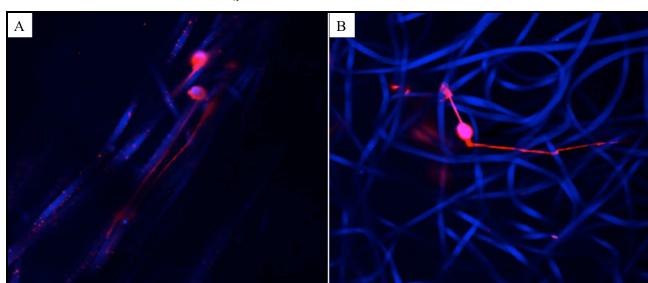
MTS assays demonstrated that the scaffolds presented excellent biocompatibility. PC-12 cells grew and proliferated well on all scaffolds (Figure 6). After 4 and 6 days culture, the cell proliferation on nanosphere incorporated PCL scaffolds were significantly higher than that other groups. The nanosphere incorporated scaffold can sustainably release BSA to support PC-12 cell proliferation. However, the BSA sprayed scaffolds released majority of BSA in the initial 10 h, which was removed by changing media.

With the introduction of NGF, PC-12 cells will stop dividing and start differentiation. Figure 7 reveals the confocal images of differentiated PC-12 cells cultured in completed neural media with 50 ng/mL NGF for 10 days on aligned and random nanocomposite scaffolds. Both

scaffolds can support PC-12 adhesion and differentiation. It is clear to see the axon outgrowth and extension. More importantly, the aligned scaffolds can direct neurite extension along the orientation of fibers (Figure 7A), while the guidance is absent on random orientated fibrous scaffolds (Figure 7B).



**Figure 6.** PC-12 cell proliferation after culturing 2, 4 and 6 days. Data are  $\pm$ standard error mean,  $n=9$ ; \* $p<0.05$  when compared to PCL with bare BSA and PCL controls on day 4 and 6.



**Figure 7.** Confocal microscopy images of PC-12 cell spreading morphology. (A) Axon extension along highly aligned fibers and (B) undirectional growth on random scaffolds (red color represents cell body, and blue color represents cell nuclei and partly stained PCL fibers).

The confocal images results showed that the topography of scaffolds can influence the differentiated PC-12 cells. There are multiple adhesion sites on random orientation fibers which makes PC-12 cells grew without uniform distribution. On the contrary, the aligned scaffolds provide contact direction for the neurite extension along the direction of fibers. The study has illustrated it is required to reduce branching of neurites and increase their length to prevent formation of neuroleoma for successful neural repair [13]. Our highly aligned scaffolds inhibited the neural cell branching and enhanced the neurite growth through contact guidance.

#### 4 CONCLUSIONS

A novel nanocomposite scaffold including a highly aligned PCL microfibrous framework with adjustable bioactive factor embedded PLGA nanospheres was fabricated by electrospinning and electrospraying

techniques. The BSA loaded PLGA nanosphere created a more hydrophilic surface and improved the scaffold's mechanical properties. The fabricated nanocomposite scaffolds can guide neural cell growth and neurite extension, deliver BSA for a longer term and greatly enhance PC-12 cell proliferation, thus hold potential for improving nerve tissue regeneration.

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