Fabrication and cellular compatibility of aligned poly(hydroxy alkanoate) nanofibers for nerve tissue engineering

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Abstract: The ability to fabricate aligned nanofibers may open new avenues for the development of nerve regeneration using tissue engineering scaffolds. In this study, aligned poly(3-hydroxybutyrate)/poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHB/PHBV) nanofibrous scaffolds were fabricated using electrospinning for the culture in vitro of Schwann cells (SCs) that physiologically assist in directing the growth of regenerating axons. The biocompatibility of the fabricated scaffolds was evaluated in terms of SCs adhesion and proliferation by DNA and metabolic activity quantification assays. In addition, scanning electron microscopy (SEM) and immunostaining analysis was performed to assess cell morphology and functionality. The results of this study showed that cells attached, proliferated and were metabolically active on scaffolds during 2 weeks of culture and positive for the SCs marker. Furthermore, SCs cultured on aligned nanofibers exhibited enhanced unidirectional cells orientation along the orientation of nanofibers and significantly higher proliferation and metabolic activity than those cultured on random nanofibers.

Keywords: Aligned nanofibers; Scaffold; Schwann cells; Tissue Engineering

Introduction
Neural injuries are very common in clinical practice and may lead to permanent disabilities in patients. Nerve tissue engineering is a very promising way to replace the traditional autologous nerve graft, and can be facilitated by combining scaffolds and Schwann cells (SCs). SCs are responsible for the supportive environment of nerves as they produce extracellular matrix molecules, integrin and trophic factors and form longitudinally aligned strands that guide axonal regeneration [1, 2]. An ideal scaffold onto which SCs attach, proliferate, and migrate plays a key role in neural tissue engineering [3]. Highly porous electrospun nanofiber matrices can provide an ideal background for this purpose, due to physical and structural similarities to ECM components such as collagen fibers and their high surface area [4]. It has also been reported that alignment of nanofibers help in providing guidance cues to the extension of Schwann cells and regeneration of axons [5]. Various synthetic and natural polymer fibers with fiber diameters ranging from tens to hundreds of nanometers have been fabricated and used for nerve tissue regeneration. Poly (3-hydroxybutyrate) (PHB) and Poly (3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) are the most widely-used members of Polyhydroxyalkanoates (PHAs) for various biomedical applications due to their inhere biocompatibility and biodegradability. These polymers have also been studied as an implant material to guide axonal growth after nerve injuries [1].

In this study, we examined the effect of fiber alignment of PHAs electrospun nanofibers on the response of SCs in terms of their metabolic activity, proliferation, morphology, and functionality. Aligned and random nanofibers have been created by varying the speed of rotating mandrel collector using an electrospinning setup.

Experimental
A polymeric solution (6% wt/wt) of PHB (50) / PHBV (50) (Sigma-Aldrich) blend was prepared by dissolving the two components in a mixture of chloroform and dimethylformamide (9:1 v/v) solvents. The desired solution was loaded into a syringe and fed using a pump (KDS 100, KD Scientific) at a rate of 1.5 ml/min. The other end of the syringe was connected to a needle, on which a positive voltage of 16 kV was applied using a high voltage generator (Gamma High Voltage Research Inc., USA). A rotating mandrel collector placed 15 cm from the needle was used with speed of 500 and 5000 rpm to collect random and aligned nanofibers, respectively. The morphology of PHB nanofibers was observed by scanning electron microscopy (SEM) and fiber diameters were measured with a manual microstructure distance measurement.

The in vitro experiments were performed with a rat Schwann cell line RT4-D6P2T (ATCC, USA), cultured in DMEM high glucose, supplemented with 10% fetal bovine serum (FBS) and 1% Penicillin/Streptomycin. After sterilization, each electrospun disc (diameter = 15 mm and thickness= 240 ±13 μm) was seeded with 50’000
cells. On days 1 and 7, one sample from each group was used for SEM analysis. The cells were fixed in 4% formalin for 30 min and dehydrated in a series of increasing ethanol concentrations, before being dried in a critical point dryer (Balzers CPD-030). Samples were then sputter coated with gold (Cressington) for observation on the SEM.

Cell metabolism was assessed using a Presto blue assay (Biosource, Camarillo, CA, USA) according to the manufacture’s protocol. Briefly, culture medium was replaced with medium containing 10% (v/v) Presto blue solution and the cells were incubated at 37 °C for 2 h. Fluorescence was measured at 590 nm on a Perkin Elmer Victor3 1420 Multilabel plate reader. Cell metabolism was analyzed on day 1, 3, 7 and 14 (n=3).

After 1 and 14 days of cell culture, the scaffolds were taken from the culture medium, washed in PBS and were digested at 56 °C for at least 16 h in a Tri-EDTA buffered solution containing 1 mg/ml Proteinase-K, 18.5 µg/ml pepstatin A, and 1 µg/ml iodoacetamide (Sigma-Aldrich). DNA assay was performed with Cyquant dye kit according to the manufacturers description (Molecular Probes, U.S.A.), using a spectrophuorometer (Victor3, Perkin Elmer, U.S.A.), at an excitation wavelength of 480 nm and an emission wavelength of 520 nm.

For immunostaining, the cells fixed with 4% (v/v) paraformaldehyde (Sigma) in PBS for 30 min. Fixed cells were blocked with 10% wt/v BSA in PBS blocking buffer for 1 h. Primary antibodies against p75 low affinity NGF receptor (p75LNGFR, 1:500, Abcam; ab6172) were applied in dilute buffer consisting of 10% BSA in PBS overnight at 4 °C. Cells were washed and the secondary antibody, goat anti-mouse IgG conjugated-fluorescin isothiocyanate (FITC, 1:50), was applied for 45 min at 37°C. Cells were counterstained with DAPI and observed under fluorescence microscope. For negative controls, the primary antibody was excluded.

Results and Discussion

The morphology of aligned and randomly oriented fibers was examined by SEM. As shown in Figure 1, randomly oriented and uniaxially aligned fibers had mean diameters of 856± 175 and 879±276 nm, respectively.

To evaluate the influence of sample topography on SCs metabolic activity and cell proliferation, SCs were cultured for 14 days on PHB/PHBV random and aligned nanofibers. SCs metabolic activity increased with time in all scaffolds and was significantly higher on the aligned fibers than on the randomly oriented one, on days 7 and 14 (Figure 2A). The DNA quantification results (Figure 2B) helped to better understand the SCs proliferation on scaffolds during the 14 days of cell culture. From day 1 to day 14, DNA amount increased for each scaffold. After 2 weeks a significant difference between cell proliferation on aligned and randomly oriented nanofibers was measured.

![Figure 1](image1.png)

![Figure 2A](image2A.png)

![Figure 2B](image2B.png)

Although the in vitro assays above illustrated the cellular biocompatibility of the fabricated scaffolds, direct cellular interactions with the material surface also plays an important role in tissue regeneration. For example, SCs morphology and directionality is a key contributing factor to neuritogenesis [6]. Therefore, the influence of aligned PHB/PHBV nanofibers on regulating cell adhesion and spreading was investigated by incubating SCs on aligned and randomly oriented fibers. SCs cultured on the materials for 1 nad 7 days were examined with SEM. As shown in Figure 3, SCs grown on different PHB/PHBV samples exhibited substantially different morphologies. SCs on the randomly oriented fibers had multiple focal adhesions resulting in a multipolar morphology and flatter cell bodies. Alternatively, SCs on the aligned PHB/PHBV nanofibers appeared to interact with individual aligned fibers, exhibiting an extended, bipolar morphology that oriented along the fiber direction, which enhanced cell alignment. This was consistent with cellular biological analysis (Figure 2), where cells showed enhanced metabolic activity and proliferation on aligned fibers. It is reported that, aligned
Electrospun fibers showed to effectively direct SCs and neurite extension from primary cell explants and within nerve injury models [7]. Similarly, the aligned fibers produced in this study effectively directed SCs activity without requiring a further surface biological functionalization.

P75LNGFR is a common cell marker for Schwann cells [8]. Immunocytochemistry indicated that cultured SCs were also positive for p75LNGFR, thus confirming cell functionality. Furthermore, fluorescent microscopy also confirmed spread cell morphology and tight attachment to the electrospun mat (Figure 4).

**Conclusions**

Aligned nanofibers provide a favorable environment for Schwann cell proliferation and function. The highly aligned PHB/PHBV nanofibrous scaffolds were shown to direct SCs attachment resulting in a characteristic bipolar cell morphology necessary for nerve regeneration as compared to randomly oriented fibers. Fiber alignment also enhanced SCs metabolic activity and proliferation, demonstrating the increased cellular responses induced by oriented fiber topographies. Our study suggests that the PHB/PHBV aligned nanofibers may elicit topographical cues for the attachment and growth of Schwann cells and could serve as a potential scaffold for nerve regeneration.

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**References**


