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Carbon-nanotube-interfaced glass fiber scaffold for regeneration of transected sciatic nerve

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ABSTRACT

Carbon nanotubes (CNTs), with their unique and unprecedented properties, have become very popular for the repair of tissues, particularly for those requiring electrical stimuli. Whilst most reports have demonstrated in vitro neural cell responses of the CNTs, few studies have been performed on the in vivo efficacy of CNT-interfaced biomaterials in the repair and regeneration of neural tissues. Thus, we report here for the first time the in vivo functions of CNT-interfaced nerve conduits in the regeneration of transected rat sciatic nerve. Aminated CNTs were chemically tethered onto the surface of aligned phosphate glass microfibers (PGFs) and CNT-interfaced PGFs (CNT–PGFs) were successfully placed into three-dimensional poly(L/D-lactic acid) (PLDLA) tubes. An in vitro study confirmed that neurites of dorsal root ganglion outgrew actively along the aligned CNT–PGFs and that the CNT interfacing significantly increased the maximal neurite length. Sixteen weeks after implantation of a CNT–PGF nerve conduit into the 10 mm gap of a transected rat sciatic nerve, the number of regenerating axons crossing the scaffold, the cross-sectional area of the re-innervated muscles and the electrophysiological findings were all significantly improved by the interfacing with CNTs. This first in vivo effect of using a CNT-interfaced scaffold in the regeneration process of a transected rat sciatic nerve strongly supports the potential use of CNT-interfaced PGFs at the interface between the nerve conduit and peripheral neural tissues.

1. Introduction

Peripheral nerve injury is frequently encountered in the clinical setting. An injured peripheral nerve can regenerate spontaneously, but the regenerative capacity is limited in long defects and severe injury [1]. Current medical and surgical management techniques, including autologous nerve grafts and allografts, are in most cases not sufficient for complete regeneration of the damaged peripheral nerve [2]. Artificial nerve conduits, such as single hollow tubes, are commercially available for the connection of transected peripheral nerves, but are not thought to be suitable as a physical guide for the regeneration of a long defect [3]. Many types of scaffold configuration and fabrication, including intraluminal microchannel formation [4] and electrospun nanostructured scaffolds [5,6], have been attempted to give physical and biological cues for outgrowing axons and to overcome the limitations of regeneration in the peripheral nervous system. The delivery of growth factors [7], pharmacological agents [8], stem cells [9] or Schwann cells [10] within the nerve conduit might be other options for improving neural regeneration [11,12].

Intraluminal structures for physical guidance of outgrowing axons have been developed using collagen fibers [13], denatured muscle tissue [14] and aligned phosphate glass fiber (PGF) bundles [15], though the results thus far have proved unsatisfactory.

Carbon nanotubes (CNTs) have unique chemical, mechanical, structural and electrical properties that make them attractive for the repair and regeneration of tissues, including nerves, and functionalized CNTs have also been applied to stroke and spinal cord injury models [16–18]. A body of key literature has already demonstrated the significant and profound effects of CNTs,

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particularly on nerve cells and even stem cells, with regard to their neurite outgrowth and neuronal differentiation [19–23], and CNT-based substrates have been suggested as potential agents for the stimulation of neuronal functions and the repair and regeneration of damaged and diseased neural tissues [18,24]. The nanotopographical and biochemical features and electrical conductivity of CNTs may mediate neural modulation [25]. Therefore, CNTs are expected to have synergistic effects on peripheral nerve regeneration when interfaced with an intraluminar structured scaffold. However, most of the studies mentioned were performed in vitro, and there is little evidence about the in vivo functions of CNT-interfaced biomaterials in nerve damage models.

Therefore, we show here for the first time the in vivo effects of CNT-interfaced substrates on nerve regeneration using a transected rat sciatic nerve model. For this, we chemically linked functionalized CNTs onto the surface of aligned PGF bundles, aiming at utilizing CNTs as an interfacing material while the aligned fiber bundles are expected to function for physical guidance. Our previous studies on PGF have shown that aligned PGFs within a collagen scaffold were effective in guiding nerve tissues in a transected rat sciatic nerve model as well as in a transected rat spinal cord injury model [15]. PGFs, a class of optical glasses composed of metaphosphates of various metals, offer biocompatibility and tailored directionality; as such, they are considered to be suitable for the regeneration of tissues requiring directional guidance, including muscle and nerve [15,26,27]. We implanted a CNT-interfaced PGF neural scaffold in a 10 mm transected sciatic nerve for 16 weeks and the effects on axonal guidance, reinnervation of muscles and the electrophysiological functions were delineated and compared with the findings for a non-interfaced PGF scaffold. It is hoped that this first in vivo study using a CNT-interfaced biomaterial scaffold will provide some informative and pioneering concepts on the possible utility of CNT interfacing as a novel guide and scaffold for the repair and regeneration of nerve tissues.

2. Materials and methods

2.1. Preparation of CNT–PGFs and nerve scaffolds

The composition of phosphate glass was P_2O_5–CaO–Na_2O–Fe_2O_3, with a 50–40–5–5 mol.% ratio. The generation of microfiber bundles of the phosphate glass has been described in detail elsewhere [15]. Produced microfibers were aligned using a microcomb, fixed on one end with heat-melted poly(caprolactone) (PCL; Sigma–Aldrich, St. Louis, MO, USA) solution and then dried. The aligned microfibers were cut to a length and width of about 18 mm, then fixed on the other end with PCL, which can be directly applied in both in vitro and in vivo experiments. Together with the microfiber form, a disc of the phosphate glass was also prepared for characterization of the surface modification of the phosphate glass, after sintering phosphate glass powder of the same composition.

The aligned PGF bundle was interfaced with CNTs, so that it could play the role of a guiding substrate for the neural cells, as depicted in Fig. 1A. The series of chemical reactions for this CNT tethering is shown schematically in Fig. 1B–D. First, the glass surfaces were positively charged with amine residues. The glass microfiber bundles and discs were pretreated with 1 N hydrochloric acid for 5 min, treated with 2.5% 3-aminopropyl-triethoxysilane (APTES; Sigma–Aldrich) at pH 5.0 for 10 s, then dried with a heat gun (~120 °C) 10 times (Fig. 1B). CNT solution was prepared after carboxylation of raw CNTs by the acid oxidation method. Briefly, 0.5 g of CNTs (multi-walled, 15–20 nm outer diameter, 10–20 μm length; EM-Power Co., Asan, Korea) was added to H_2SO_4/HNO_3 1:1 aqueous solution and refluxed at 80 °C for 2 days, followed by filtration through a 0.4 μm Millipore membrane. The resultant carboxylated CNTs were washed and dried under a vacuum, then dissolved in ethanol to a concentration of 0.0025 wt.%. The aminated glass bundles and discs were then soaked in the CNT–COOH solution with 0.006 mM 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC; Sigma–Aldrich) at room temperature for 3 h to enable amide bonds to form (Fig. 1C). The CNT–PGF surface was further functionalized with amine groups by carbodiimide crosslinking with 0.1 M ethylenediamine (Sigma–Aldrich) and 0.012 mM EDC at pH 5.0 and room temperature for 2 h to leave amine groups at the surface of the CNT–PGF substrate (Fig. 1D). Samples were rinsed with a series of ethanol solutions and distilled water (DW) to remove excess chemical byproducts, before being sterilized first in 70% ethanol and then under UV irradiation for further biological assays.

The aminated CNT–PGF substrate was then incorporated into cylindrical nerve scaffolds. The scaffolding of the microfiber bundles was carried out as a two-step process: first wrapping them around a biopolymer nanofiber mat (Fig. 1E) and then placing it within a porous biopolymer cylindrical tube (Fig. 1F). First, a PLDLA electrospun nanofiber mat was prepared. PLDLA solution in chloroform (2.5 wt.%) was electrospun onto a high-speed rotating metal collector to gather up aligned PLDLA nanofibers. The electrospinning conditions were a 1.5 kV cm^{-1} electric field strength and a 0.1 ml min^{-1} injection speed. The microfiber bundles were placed onto the nanofiber mat, which was then rolled up to wrap (three times) the bundles completely. The number of microfibers wrapped within the nanofiber mat was determined to be 900 ± 36. The nanofiber-wrapped microfiber bundles were then placed within a PLDLA cylindrical tube. The PLDLA tube was produced by the method described elsewhere with a slight modification [28]. In brief, 0.2 g of PLDLA and 1 g of ionic liquid ([bmim]BF_4) were dissolved in 10 ml of dichloromethane, in which a glass tube (0.8 mm diameter) was immersed to coat it with a thin layer (~200 μm) of the PLDLA–ionic liquid. After completely drying, the ionic liquid was selectively dissolved in DW by gentle washing, to leave a porous structured PLDLA cylindrical tube.

2.2. Characterization of CNT–PGFs and scaffolds

The identification and quantitative analysis of chemical reaction were accomplished with a zeta potential analyzer (Zetasizer Nano ZS, Malvern Instruments Ltd., Worcestershire, UK), Fourier transform infrared spectrometry (Varian 640-IR, Varian, Palo Alto, CA, USA), X-ray photoelectron spectroscopy (XPS; AES-XPS ESCA 2000, Thermo Fisher Scientific Inc., Waltham, MA, USA) and thermogravimetric analysis (TGA; TGA N-1500, Scinco, Seoul, Korea). The morphology of the samples was examined by field emission scanning electron microscopy (FESEM; MIRA II LMH microscope, Tescan, Czech Republic) and transmission electron microscopy (TEM; JEM 2000EXII, Jeol Ltd., Tokyo, Japan). The water wetting property of the samples was examined by contact angle analysis (Phoenix 300, Surface Electro Optics, Gyunggido, Korea). The electrical conductivity was analyzed using a high-resistance measurement (Agilent 4339B/4349B, Agilent Technologies, Inc., Santa Clara, CA, USA).

The physical and chemical stability of the CNTs linked to the PGF surface were examined. For the physical stability, microfiber bundles were treated with ultrasound for 10 min, after which the CNTs' existence and status on the surface were observed by FESEM. The chemical stability was observed by soaking the sample in DW for periods of up to 28 days. At predetermined times, the sample was taken out and the surface status was examined by FESEM.

2.3. In vitro study of CNT–PGFs using PC12 and DRG cells

For the in vitro study, aligned microfiber bundles (either PGFs or CNT–PGFs) were used by fixing the ends of bundles with PCL