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Establishment of nerve growth factor gradients on aligned chitosan-polylactide /alginate fibers for neural tissue engineering applications

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ABSTRACT

Nerve conduits containing aligned fibrous fillers with gradiently distributed signal molecules are essential for long-gap nerve repair. This study was to develop an approach for establishing nerve growth factor (NGF) gradients onto the aligned chitosan-polylactide (CH-PLA) fibers. CH-PLA containing 37 wt% of PLA was spun into fibers using a wet-spinning technique. CH-PLA fibers showed much higher wet-state tensile strength, enhanced degradation tolerance and significantly lower swelling degree in comparison to chitosan fibers. The CH-PLA fibers with diameters from 40 to 60 μm were selected and segmentally coated in bundles using NGF-contained alginate solutions to establish NGF gradients lengthwise along fibers. The diameter of resulting NGF-loaded CH-PLA/alginate fibers was well controlled within a range between 60 and 120 μm. Calcium ion crosslinked alginate coating layers on fibers showed abilities to administer the sustainable NGF release in a gradient distribution manner for at least 5 weeks. NGF-induced neurite outgrowth of PC12 cells confirmed that bioactivity of NGF released from fibers was well retained.
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1. Introduction

Peripheral nerve injuries often occur as a result of traumas, invasive surgical procedures and nerve-related diseases [1]. The severe nerve injuries involving in full disruption of the neural tube frequently result in the formation of nerve gaps [2]. In the case of short gap, anastomosis of nerve fascicles can be implemented by direct suturing [1,2]. With regard to larger nerve gaps, commonly referring to a gap longer than 1 cm, nerve grafts are usually used. For long-gap nerve repair, nerve autografting would be an ideal approach, but its application is significantly limited due to its inherent drawbacks, including limited availability, a need for additional surgical interventions, possible morbidity at donor sites, and the mismatch in size and structure of donated nerves [2–4].

Artificial nerve conduits have attracted increasing research interest and clinical attention since last decade [5,6]. To date, many types of conduits have been developed by using different materials and techniques [2,3]. Despite the diversity of morphology and structure, conduits usually have lumens which are stuffed with fibers, gels or sponge-like fillers [6,7]. Among different types of fillings, aligned fibers that are longitudinally filled into the conduit are particularly conducive to long-gap nerve repair [6–9]. Studies have revealed that long fibers filled through the conduit can act as sub-level orientation substrates and direct elongation of regenerated axons, helping to organize nerve fasciculus and to facilitate nerve tracts to target the stumps at the distal end [1,6,10–15].

In addition to endowing conduit with topographical cues, addition of signal molecules to the conduit for establishing a growth-promoting milieu has also attracted considerable interest [2,7,16]. Signal molecules, including nerve growth factor (NGF), neurotrophin, brain-derived neurotrophic factor, glial cell line-derived neurotrophic factor and ciliary neurotrophic factor, have been tentatively incorporated into conduits to promote nerve growth [16]. Among them, NGF is considered as an important promoter for neuron survival, branching and outgrowth [11,16].

It has been recognized that after the occurrence of neurotmesis, growth cones generated by neurons at the proximal end would start to explore their surrounding environment as they advance [2]. During the extension of regenerated axons, contact-mediated cues with either attractive or repulsive regulatory functions will partially guide orientation of growth cones, and meanwhile, the

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concentration gradients of signal molecules will also provide spatial or directional guidance for the axon extension [16–18]. Among these signal molecules, NGF that is gradiently distributed across the nerve gap plays an important role in guiding the growth of sprouted axons [3,7,16,18]. It has been reported that growth cones of axons can orient themselves toward the NGF source and sensitively detect NGF gradients that differ by as low as 1–2% across their diameter or a NGF concentration difference as small as about one molecule across their spatial extent [16,17,19,20].

Because of the important role of NGF, nowadays, significant attention has been directed toward establishment of NGF gradients on conduits [21–33]. Signal molecules can be directly incorporated into the conduit wall in a gradient form. However, this approach shows limited efficiency in regulating the orientation and extension of axons because the factors laterally diffused from the conduit wall are unable to rebuild controllable factor gradients longitudinally along the central axis of the conduit [6,9,31]. Other types of conduit-filler constructs have thus been developed, where factor gradients were established inside the conduit by using factor-loaded sponge-like fillers, gels or microspheres [24,25,29–33]. As fibrous fillers are specially recommended to fill the conduit that will be used to bridge long nerve gaps [6–15], and thus, intraductal factor gradients should be built using factor-loaded fibers. Nevertheless, little has yet been done to establish factor gradients on fibers that are intended for long-gap nerve repair.

This study was designed to establish NGF gradients onto aligned fibers. Chitosan-polylactide (CH-PLA) fibers were adopted as a core material, and NGF was loaded onto CH-PLA fibers via alginate coating layers, with a graded concentration distribution longitudinally along the fibers from one end to another. Then the controlled release of NGF and bioactivity of the released NGF were evaluated in cell culture. Therefore, this study has provided valuable baseline information for exploring novel strategies to assemble conduit-fiber constructs for long-gap nerve repair.

2. Materials and methods

2.1. Materials and reagents

Chitosan (deacetylation degree: ca.94%, viscosity-average molecular weight: ca.5.4×10^4) was procured from Aladdin, China. NGF and ^{125}I NGF were bought from PeproTech and PerkinElmer, respectively. Sodium alginate (low viscosity), L-lactide (LA) and fluorescein isothiocyanate (FITC) labeled dextran (MW 10 kD) were purchased from Sigma-Aldrich. Lysozyme, sodium azide, fetal calf serum (FCS), penicillin, streptomycin, DMEM, sodium sulfate, sodium hydroxide, anhydrous ethanol, acetic acid, phthalic anhydride, hydrazine monohydrate, anhydrous dimethylformamide (DMF), stannous chloride and calcium chloride were purchased from Sinopharm, China.

CH-PLA copolymers were synthesized by grafting PLA side chains onto the C-6 sites of chitosan backbone using group-protection methods described elsewhere [34,35]. In brief, amino groups at the C-2 sites of chitosan were protected by reacting chitosan with phthalic anhydride, and the resulting phthaloyl chitosan (PHCH) was further reacted with LA under N_2 in the medium of DMF to synthesize PHCS-PLA copolymers. PHCS-PLAs were deprotected by eliminating phthaloyl groups using hydrazine monohydrate to achieve CH-PLAs. By mainly changing the feed ratio of LA to PHCS, CH-PLAs containing various weight percentages of PLA were synthesized. Taking into account the required solubility of CH-PLAs in aqueous media as well as the required strength and degradation properties of resulting CH-PLA fibers, the CH-PLA with PLA content of 37.4 ± 1.9 wt% was used for spinning CH-PLA fibers.

2.2. Preparation of CH-PLA fibers

A laboratory scale wet-spinning system was set up for CH-PLA fiber spinning following methods similar to those described elsewhere [36–39]. Briefly, CH-PLA was dissolved in 2.0% aqueous acetic acid to prepare a 4 wt% CH-PLA dope. By applying nitrogen pressure of around 60 kPa, the dope was extruded continuously through a volume-metering pump, a stainless steel filter and a 50-hole stainless steel spinneret (each hole:250 μm in diameter, 500 μm in length). The coagulation bath was filled with a $NaOH-Na_2SO_4$ aqueous solution in which the concentration of NaOH was 10% and the concentration of Na_2SO_4 was 20%. The formed fibers were stretched to 1.2-1.4 fold in a $NaOH-CH_3CH_2OH$ aqueous solution (NaOH was dissolved in 50% ethanol aqueous solution at a 2 wt% concentration) when they went through the stretch bath. After being washed with deionized water, CH-PLA fibers were dehydrated in a concentration-ascending methanol series, collected using a bobbin and air-dried. Chitosan fibers were also produced with the same method under the same spinning conditions.

2.3. Establishment of NGF gradients on CH-PLA/alginate fibers

A schematic illustration for establishing NGF gradients on aligned CH-PLA fibers was presented in Fig. 1. Alginate solutions containing NGF together with a ^{125}I NGF tracer (hot/cold ratio: 1/80) and $CaCl_2$ solutions were prepared, respectively, and they were filled into 10 tubes (5 mm in diameter). NGF-containing alginate solutions were filled into tubes 1, 3, 5, 7 and 9, and used to coat CH-PLA fibers, and correspondingly, $CaCl_2$ solutions were filled into tubes 2, 4, 6, 8 and 10, and used to crosslink alginate layers binding on CH-PLA fibers. 10 tubes were arranged in pairs, as shown in Fig. 1(A). The alginate concentration in tubes 1, 3, 5, 7 and 9 uniformly increased while the NGF concentration gradually decreased. On the other hand, the $CaCl_2$ concentration in tubes 2, 4, 6, 8 and 10 was maintained at three times that of the matched alginate concentration.

NGF-loaded CH-PLA/alginate fibers were prepared as follows. In a typical procedure, aligned dry CH-PLA fibers (number of fibers: 10) were dipped into tube 1 to a depth of 10 mm for 5 min, and then, lifted up and air-dried at room temperature for 30 min. After that, the alginate-coated end of fibers was dipped into tube 2 to a depth of 10 mm for 10 min, lifted up and air-dried again for another 30 min. These operations were repeated until fibers were lifted up from tube 10. The resulting NGF-loaded CH-PLA/alginate fibers were further dipped in a graded ethanol series to a depth of slightly higher than 50 mm to harden the alginate layers for 30 min, and finally, they were fully air-dried. A 10-hole fiber-passing board was used during assembly procedure to prevent fibers from sticking.

By cutting off the uncoated portion of fibers, a NGF-loaded CH-PLA/alginate fiber bundle with a length of 50 mm was achieved and the NGF load in the fiber bundle was distributed in a graded manner longitudinally along the bundle length, as indicated in Fig. 1(B).

NGF-loaded CH-PLA/alginate fiber bundle was cut into 5 segments (see Fig. 1(B)) each having a length of 10 mm, and initial NGF load and loading efficiency (LE) for these segments were determined using a gamma counter (Cobra II, Packard).

To see the formation of alginate-coating layer on CH-PLA fibers, some alginate solutions containing no NGF but loading with FITC-dextran were employed to coat CH-PLA fibers, using the same protocol applied to the preparation of NGF-loaded CH-PLA/alginate fibers, and these FITC-dextran-contained CH-PLA/alginate fibers were used for fluorescence imaging. In addition, blank alginate solutions containing neither NGF nor FITC-dextran were used to coat CH-PLA fibers to prepare blank CH-PLA/alginate fibers following the same method shown in Fig. 1(A). The blank CH-PLA/alginate

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