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PDLLA/chondroitin sulfate/chitosan/NGF conduits for peripheral nerve regeneration

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ABSTRACT

Biodegradable PDLLA/Chondroitin sulfate/Chitosan(PDLLA/CS/CHS) nerve conduits with potentially good biocompatibility and good mechanical property feasible for surgical manipulation have been developed in our previous work. The purpose of this study was to investigate their possible application in repairing damaged nerves and the effect of nerve growth factor (NGF). The PDLLA/CS/CHS/NGF nerve conduits were prepared by immobilizing NGF onto the PDLLA/CS/CHS nerve conduits with carbodiimide. Adult Sprague–Dawley (SD) rats weighing 200–250 g were used as the animal model. The conduits were employed to bridge the 10 mm defects in the sciatic nerve of the SD rats. Nerve conduction velocities (NCVs) were clearly detected in both nerve conduits after 3 months of implantation, indicating a rapid functional recovery for the disrupted nerves. The results of histological sections showed that the internal sides of the conduits were compact enough to prevent the connective tissues from ingrowth. Combined with the strong mechanical properties, good nerve regeneration ability and non-toxicity of its degradation products, PDLLA/CS/CHS nerve conduits would be expected to be useful materials to repair nerve damage and NGF can effectively promote the regeneration of peripheral nerve defect.

1. Introduction

Autologous nerve grafts have been commonly used in bridging peripheral nerve defects [1,2]. However, unavoidable disadvantages, such as the limited availability and donor-site morbidity of autografts, still remained. Although allografts have also been used, these were accompanied by the usual need for immunosuppression and have lower success rates [3]. In recent years, enormous effort has been devoted to develop the synthetic nerve conduits for the repair of peripheral nerve defects. Synthetic nerve conduits show a promise for replacing autologous nerve grafts and offer an "off-the-shelf" solution to avoid the sacrifice of a healthy nerve [4–8].

A wide range of materials has been developed for use as a synthetic nerve conduit. Of these materials, nondegradable materials, such as silicone rubber have been widely used in general clinical case because of their good inert and mechanical properties. However, upon completion of regeneration, these materials may become detrimental due to mechanical impingement, foreign body reaction or infection [8]. Contrarily, biodegradable materials potentially avoid these problems and make secondary surgery unnecessary. Different kinds of biodegradable biomaterials have been studied, such as polyglycolic acid (PGA) [2], poly (L-lactide-coglycolide) (PLGA) [9,10], poly (3-hydroxybutyrate) (PHB) [11,12], gelatin [13–15], collagen [16,17], chitosan [18,19] and silk[20,21]. However, the properties of them such as mechanical properties, processibility and biocompatibility do not fully meet the requirements of an ideal nerve conduit. For example, nerve conduits have been fabricated out of collagen with favorable results in nerve repair [16,17]. But it is rather expensive and difficult to handle during suturing, furthermore, it can produce inflammatory reactions and shown complete bioerosion within 2 months after implantation because of its mechanical weakness [22].It is believed that an ideal biodegradable conduit should involve properties to maintain its structure integrity, to allow cell-to-cell communication and subsequent tissue ingrowths during the regenerative processes [23,24].

PLA is used for tubulization to bridge both ends of the amputated peripheral nerve because of its good biodegradability, biocompatibility and mechanical properties [25,26].But its degradation by-products would lead to a lowering of local pH and could be detrimental to the surrounding cells and tissue [27,28]. Chitosan is a cationic natural biopolymer and can be used for nerve repair

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due to its good biocompatibility, biodegradability, low toxicity and low cost [18,19]. However, its clinical application is limited, because of low mechanical strength until now. Chondroitin sulfate (CS), a natural anion polymer, is an important extracellular matrix (ECM) component, which takes an important role in maintaining cell functions [29].In nature, the biological world is built up via precise self-assembly of biomacromolecules, which gives investigators, a great inspiration to explore an engineered conduit via macromolecules self-assembly. Layer-by-layer electrostatic self-assembly (LBL-ESA) of polyelectrolytes constitutes an elegant and simple approach to the design of new materials in a controlled way [30–32]. When designing a nerve guidance channel from tissue engineering view, besides conduit material, incorporation of neurotrophic factors and cells supporting axon regeneration should also be considered. Nerve regeneration has been found to be enhanced by utilizing guidance channels filled with neutrophic factors, such as nerve growth factor (NGF), glial cell line derived neurotrophic factor (GDNF) and neurotrophin NT-3 [10,20,26,33,34].

In our previous study [35,36], PDLLA/CS/CHS nerve conduits have been developed via LBL-ESA technology. The drawbacks of PLA and chitosan mentioned above have been better overcome. They are biodegradable with good biocompatibility with Schwann cells *in vitro* and good mechanical property feasible for surgical manipulation. The purpose of this study was to investigate their possible application in repairing damaged nerves and the effects of NGF immobilized onto the tube. To do this, conduits were fabricated to bridge the 10 mm defects in the sciatic nerve of SD rats, and autograft nerves were taken as positive controls and PDLLA nerve conduits were taken as controls too.

2. Materials and methods

2.1. Materials

Poly(d, L-lactic acid) (PDLLA) (Mw = 250 000) was synthesized in our laboratory, Chondroitin sulfate (CS)and Chitosan (CHS, Mw = 30 000, DD = 90%) were purchased from Sigma–Aldrich . To obtain rhodamine B isothiocyanate (Rd) labeled 2% (w/v) solution of chitosan, chitosan was stained by 2 mg/ml rhodamine B isothiocyanate solution at 4 °C for 48 h and then dialyzed with 0.1 mol/l acetic acid for 1 month. All other chemicals were analytical reagents. Adult Sprague–Dawley (SD) rats were purchased from Tongji Medicinal School, Huazhong University of Technology (Wuhan, Hubei, China).

2.2. Preparation of PDLLA/CS/CHS nerve conduits

PDLLA was dissolved in dioxane to form a 1(w/v) % polymer solution, and then a cylinder mandrel was put into it for several seconds and took it out. After dioxane being volatilized, the cylinder mandrel was put into the solution again. The core material obtained was treated in a vacuum oven until a constant weight was reached, and then washed in ethanol-water (1:1) solution for 24 h to remove the grease and other impurity on the surface. After washed with deionized water, the core material was immersed in a solution of 2 mg/ml 1, 6-hexanediamine isopropyl alcohol solution for 2 h at 40 °C, subsequently washed with deionized water to removed the unreacted 1, 6-hexanediamine and dried in vacuum oven at 30 °C for 24 h to obtain a aminolyzed core material with constant weight.

The aminolyzed core material was put in 0.02 mol/l HCl solution to react for 2 h at 25 °C, and then washed with tri-distilled water to remove the excess HCl. The acidification core material was deposited in CS solution for 1 h to get a negatively charged surface. The excess CS was removed by 0.5 mol/l NaCl tri-distilled water solution. Next, positively charged chitosan was deposited under the same conditions and procedure. The above process was repeated until the desired number of layers was deposited on PDLLA substrate. The composite material was frozen at −70 °C for 24 h and lyophilized in a vacuum freezer drier for 48 h to obtain the PDLLA/CS/CHS nerve conduits.

2.3. Monitoring of the deposition process and characterization of the nerve conduits

Instead of the common chitosan, the Rd-chitosan was used to monitor the deposition process using UV–VIS absorbance spectroscopy (UV-2550, Shimadzu, Japan). The attenuated total reflectance-Fourier Transform Infrared (ATR-FTIR) transmission spectra were realized from the sample on a Nicolet Nexus FTIR Spectroscopy (Nexus, Thermo Nicolet, American). The nerve conduit's surfaces were typically characterized by using X-ray photoelectron spectroscopy (XPS) (PHI 5300 , Perkin–Elmer, American).

2.4. Release of NGF in vitro

PDLLA/CS/CHS/NGF nerve conduits were prepared by immobilizing NGF onto the PDLLA/CS/CHS nerve conduits. The conduits were soaked in 20 mL, 0.1wt% 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDAC) solution for 24–48 h at 0–4 °C , and then transferred to 5 mL, 20 ng/mL NGF solution and soaked for 24–48 h at 0–4 °C. The un-reacting NGF and excessive chemicals were removed by washing the conduits with the phosphate buffer solution (PBS, pH = 7.4), the conduits were then lyophilized in a vacuum freezer drier. The NGF release properties *in vitro* were carried out in 2 mL PBS (37 °C, pH = 7.4) ($n = 5$). At each time point, the release solution was taken out and the corresponding proportion fresh PBS solution was added. The collected release solution was saved at a low temperature and determined on a Multiskan Spectrum enzyme sign meter (Multiskan FC, Thermo Lab systems , America) with Enzyme-Linked Immunosorbnent Assay (ELISA) method.

2.5. Animals and surgical procedure

Adult SD rats weighing 200–250 g were used to evaluate the nerve regeneration performance of PDLLA/CS/CHS conduits. The animals were divided into 4 groups each with 15 rats. 10 mm defects in the sciatic nerve created by surgical removal of the nerve tissue were repaired with the nerve conduits: Group A: PDLLA/CS/CHS/NGF conduits; group B: PDLLA/CS/CHS conduits; group C: PDLLA conduits; group D: autograft nerve group as a positive control. The animals were anesthetized with 50 mg/kg body weight pentobarbital sodium. The right sciatic nerve was exposed after the skin incision, and separation of muscles around the nerve tissues using blunt dissection. Subsequently, the right sciatic nerve was severed into proximal and distal segments at the center of the right thigh. Both the proximal and the distal stumps were secured with 8-0 nylon to a depth of 1 mm into the conduits, leaving a 10-mm gap between the stumps. The muscle layer was re-approximated with 4-0 chromic gut sutures, and the skin was closed with 2-0 silk sutures. Each rat received one implant, which was removed at various time intervals: namely, 3 and 6 months, respectively. At each time interval, electrophysiological and histomorphometric evaluations were performed to evaluate the efficiency of nerve conduits for nerve regeneration.

2.6. Electrophysiological assessment

Three and six months after implantation, electrophysiological tests were performed on animals, respectively. Under anesthesia, the right sciatic nerve was exposed and nerve stimulation electrodes were placed at the proximal part of the proximal joint access and recording electrodes placed in the lower leg triceps. Nerve conduction velocities (NCVs) were recorded on the lower leg triceps.

2.7. Histological assessment

The implanted conduits were harvested immediately after recording the NCVs. The nerve grafts were at the same time fixed in a cold buffered 3% glutaraldehyde solution. Nerve grafts were then washed in phosphate buffer solution (PBS) and the sciatic nerve sections were then taken from the middle regions of the regenerated nerve. After fixation, some tissues in each group were embedded with olefin and cut to 4-mm thickness and stained with hematoxylin/eosin. The other samples were embedded with Epon 812 epoxy resin and stained with methylene blue. All nerve sections were observed under a light microscope (TE2000-U, Nikon, Japan). An image analysis system (Image-Pro Plus, Media Cybernetics, America) was used to analyze the photographs for determination of the number and areas of individual myelinated axons.

The cryostat sections of the residual regenerated nerve tissue were used for the S-100 immunohistochemistry analysis. After soaking in 0.01 M PBS for 10 min and blocked in a 5% normal goat serum for 60 min at room temperature, the nerve cryostat sections were incubated with rabbit anti-mouse S-100 antibody (N4142, Sigma, Saint Louis, USA) at 4 °C for 24 h and subsequently reacted with the fluorescein isothiocyanate (FITC) labeled secondary antibody goat anti-rabbit Ig G (N4142, Bioss, Beijing, China) for 2 h at room temperature. The stained sections were observed under a fluorescent microscope (C-SHG1, Nikon, Japan).

Electron microscopy was employed to evaluate the myelin sheath regeneration. Ultra thin sections of the regenerated nerve tissues were stained with lead citrate and uranylacetate, and then were examined under a transmission electron microscope (TEM) (JEM-1200 EX, JEOL, Japan).

2.8. Statistical analysis

All numerical data were given as mean ± standard deviation. Significant differences among groups were analyzed by a one-way analysis of variance (ANOVA) followed by Tukey's post hoc test using SPSS 11.5 software for windows student version. Statistically significant value was defined as $p < 0.05$.