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ORIGINAL RESEARCH PAPER

Physical properties and biocompatibility of a porous chitosan-based fiber-reinforced conduit for nerve regeneration

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Abstract Porous fiber-reinforced chitosan nerve conduits were fabricated from chitosan yarns and a chitosan solution by combining an industrial braiding method with a mold casting/lyophilization technique. The conduits were permeable to molecules ranging in molecular size from 180 Da (glucose) to 66,200 Da (BSA). The compressive load of the reinforced conduits was significantly higher than that of a non-reinforced control conduit at equal levels of strain. The tensile strength of the reinforced conduits was also increased from 0.41 ± 0.17 to 3.69 ± 0.64 MPa. An in vitro cytotoxicity test showed the conduits were not cytotoxic to Neuro-2a cells. Preliminary in vivo implantation testing indicated that the conduits were compatible with the surrounding tissue.

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Introduction

Nerve gap repair is usually accomplished by anastomosis of the severed nerve when the two sides can be approximated without tension. However, in the case of a gap that cannot be sutured directly, the transplantation of an autologous nerve graft represents the current gold standard for nerve restoration. Nerve conduits offer a promising alternative to conventional treatments, obviating the sacrifice of a healthy nerve. They also provide physical guidance for the axons and prevent the ingrowth of scar-forming tissue into the interstitial site. Nerve conduits must fulfill several requirements: (i) they should be biocompatible, (ii) have sufficient mechanical stability during nerve regeneration, (iii) be porous enough to ensure a supply of nutrients, and (iv) should degrade to non-toxic products after bridging the gap, thus avoiding compression of the regenerating nerve tissues.

Chitosan, a natural resorbable biocompatible polysaccharide, has been considered for nerve conduit fabrication. Chitosan and chitosan-derived materials promote adhesion, survival and neurite outgrowth of nerve cells in vitro (Gong et al. 2000) and that chitosan nerve conduits supported axon elongation and functional improvement during nerve regeneration in vivo (Patel et al. 2006). Porous chitosan

scaffolds with controllable microstructures are commonly prepared by controlled freezing and lyophilizing of chitosan solutions or gels (Madihally and Matthew 1999). Recently, a mold-casting/lyophilization method was used to produce porous chitosan nerve conduits (Wang et al. 2006b). However, these scaffolds have a low mechanical strength under physiological conditions, thus limiting their applicability. In order to obtain adequate mechanical strength, chitosan conduits have been reinforced with additives (Yang et al. 2004) or crosslinked with chemical substances such as formaldehyde (Wang et al. 2005b).

In this study, we describe a method of preparation of chitosan nerve conduits that introduces braided chitosan fibers to reinforce the porous structure. These fibers are non-cytotoxic, biocompatible and can be fabricated into tubular scaffolds (Wang et al. 2005a). In combination with a mold-casting/lyophilizing method, it is feasible to provide good controllability of the scaffold properties such as conduit 3D configuration and permeability, which are important for nerve conduits. These conduits fulfilled the mechanical requirements without further additives or chemical crosslinking. The physical properties and biocompatibility of the conduits were also evaluated.

Materials and methods

Materials

Chitosan (degree of deacetylation = 85.1%, M_η = 1.8×10^6) was purchased from Qingdao Haisheng Co. (China). Chitosan solution was prepared by dissolving chitosan in acetic acid solution (1.2%, v/v), filtering it through a porous nylon cloth to remove insoluble substances, and degassing the solution under low pressure. Chitosan yarns consisting of fibers about 12 μm diam were purchased from Qingdao Jifa, Co. (Qingdao, China). All other reagents were of analytical grade.

Preparation of chitosan nerve conduits

Fiber-reinforced chitosan nerve conduits were produced with a braiding method in combination with a mold casting/lyophilizing method. The casting mold included a coping, a pedestal and a stainless steel outer tube (Wang et al. 2006b). Briefly, chitosan yarns were fabricated into hollow tubes with using a flexible industrial braiding process (Wang et al. 2006a). A segment of the braided tube was then immersed in chitosan solution (2%, w/v) at room temperature and a mandrel (a stainless steel rod coated with Teflon) was inserted into the tube. Then, the tube with the mandrel was put into the inner space of the mold, which was pre-filled with chitosan solution. The whole assembly was placed at −20°C for 12 h. The stainless steel outer tube was removed and the frozen construct was lyophilized. The samples were then immersed into 2% (w/v) NaOH and equilibrated for 30 min to neutralize the acetic acid, rinsed with distilled water until neutral, and air dried. Conduits without a fiber-reinforced structure were fabricated with chitosan solution only in the same way as described above. The inner diameter and wall thickness of the conduits can be easily controlled by varying the braiding parameters and mold dimensions.

Morphology characterization

Conduit morphology was characterized using a scanning electron microscope (SEM; KYKY-2000, China) at an accelerating voltage of 25 kV. The samples were coated with gold in vacuum prior to observation.

Permeability test

Glucose, lysozyme, and BSA were used as model molecules to test the permeability of conduits. A segment of conduit (4 mm in inner diameter and 1.5 cm in length) was sealed vertically in a well of a 24-well tissue culture cluster. About 120 μl of model molecule solution (1 mM in PBS for each) was added into the lumen of the conduit, and 2.4 ml PBS was added into the well around the conduit. The samples were placed on a shaker at 37°C. The incubation medium was collected at different time points and the diffusion rate of the glucose and proteins across the conduit wall was analyzed using a glucose estimation technique and BCA assay.

Mechanical properties

The mechanical properties of the conduits were evaluated with a universal testing machine equipped

with a 50-N load cell (AG-1, Shimadzu, Japan). All the samples were tested in a wet state after immersion in PBS for 24 h. A force was applied perpendicular to the axis of the sample with the cross-head speed of 1 mm/min. The loads were recorded at strains of 20%, 40%, and 60%. Tensile strength was tested by applying a force parallel to the axis of the conduit at an extension speed of 3 mm/min with a grip distance of 25 mm. Six samples were tested in each group.

In vitro cytotoxicity study—direct contact test with neuronal cells

Neuro-2a cells were cultured on sections of the chitosan nerve conduits in order to study cell morphology, attachment, and proliferation. The cells were cultured in a medium of 47.5% Dulbecco's modified Eagle's medium (DMEM) and 47.5% OPTI-MEM supplemented with 5% (v/v) fetal bovine serum. The conduit sections were sterilized with 75% (v/v) ethanol for 24 h, rinsed in PBS, and then placed in a 24-well tissue culture cluster. Cells were seeded directly onto the sample slices with 5×10^4 cells in each well. The medium was changed every the other day. After incubation at 37°C, 5% CO_2 for 1 d and 10 d, the samples rinsed with PBS, fixed in 4% (v/v) glutaraldehyde, dehydrated in graded ethanol and then observed under SEM.

In vivo biocompatibility study

Twelve adult female Wistar rats weighing 200–250 g were used to evaluate the biocompatibility of the conduits. The rats were anesthetized by intraperitoneal injection of sodium pentobarbital (50 mg/kg). One conduit segment (5 mm in length, sterilized in 75% (v/v) ethanol before use) was implanted subcutaneously on each side of the back of each rat. Three rats were sacrificed at each time point: 3 days and 2, 4, and 8 weeks after implantation. The implants were retrieved and fixed in 4% (v/v) paraformaldehyde, washed in water, dehydrated in a series of ethanol and embedded in paraffin. Cross sections (6 μm in thickness) were prepared and stained with hematoxylin and eosin. The tissue reaction to the implants was evaluated for uniformity and thickness of the foreign body capsule as well as inflammation under optical microscopy.

Results and discussion

Morphology characterization

The SEM results showed that the conduits possessed a well-defined and well integrated three-ply structure, including a lumen layer, a middle fiber-reinforced layer and an exterior porous layer (Fig. 1A). The exterior surface of the conduit was composed of an interconnected porous microstructure (Fig. 1B). An interconnected pore structure can increase the vascularization and nutrient exchange between the lumen and the outer environment and can also accelerate conduit degradation and integration with surrounding tissue. The pore structure of the conduits can be controlled by varying parameters of the freezing and

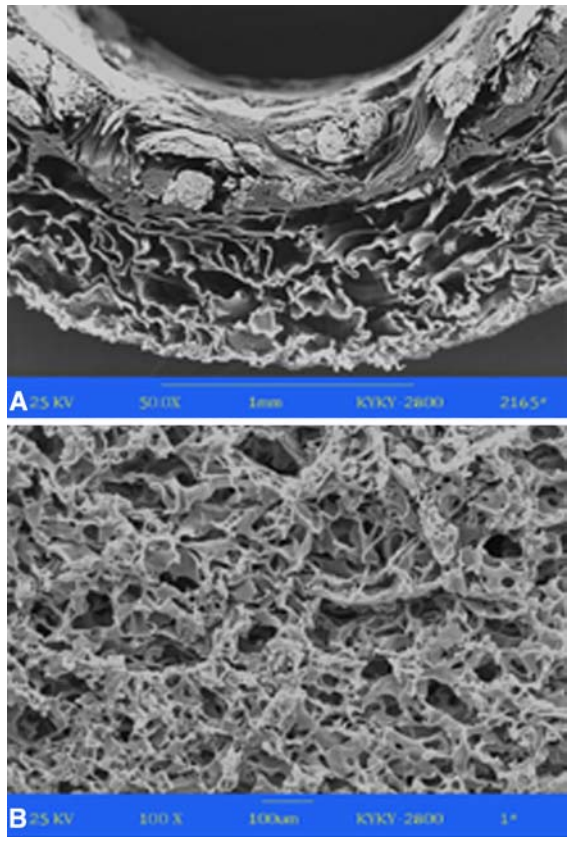

Fig. 1 SEM of fiber-reinforced chitosan porous nerve conduits. Examination of the cross-sectional morphology (**A**) showed that the conduits possess a well-integrated three layer structure. The exterior surface of the conduit (**B**) was composed of well-interconnected porous structure. Scale bars are 1 mm (**A**), 100 μm (**B**), respectively

lyophilizing processes, including solution concentration as well as freezing rate and temperature. The surface of the lumen was seen to be smoother than that of the exterior. A smooth lumen surface has been shown to be beneficial for fibrin matrix arrangement and nerve cable formation (Aebischer et al. 1990).

Permeability of the conduit

Conduit permeability is important to the transport of nutrients and waste products in vivo. The permeability of molecules, including both carbohydrates and proteins, ranging in molecular weight from 180 Da to 66,200 Da across the conduit wall are shown in Fig. 2. The conduits were permeable to all of the molecules tested, including glucose, lysozyme, and BSA, which suggests that molecules or trophic factors of similar size in body fluid could diffuse freely across the conduit. An increase in the permeability of nerve conduits is known to improve the regeneration success rate over longer nerve gaps (Jenq and Coggeshall 1987; Rodriguez et al. 1999).

Mechanical properties

Assessment of the mechanical strength of the conduits was essential to ensure that the conduits could withstand suturing and remain intact after surgery. The results showed that incorporating braided chitosan fibers into a chitosan porous structure enhanced the mechanical strength of the conduit. At the same transformation ratio, the applied force to the fiber-reinforced conduits was significantly higher than that applied to the unreinforced conduits (Fig. 3), indicating that the reinforced conduits have a higher compressive strength. Furthermore, the tensile strength of the fiber-reinforced conduit was shown to be 3.69 ± 0.64 MPa (Mean ± S.D., $n = 6$). This was also significantly higher than that of unreinforced porous conduits (0.41 ± 0.17 MPa, Mean ± S.D., $n = 6$) ($P < 0.01$).

In vitro cytotoxicity study–direct contact test with neuronal cells

The morphology of the Neuro-2a cells cultured on the reinforced conduits for 1 day and 10 days is shown in Fig. 4. The conduits showed no obvious effects on cell growth or proliferation.

In vivo biocompatibility study

No systemic or regional surgical complications were seen for any of the rats in the post-operative period. Throughout the study period, the conduits maintained lumen and wall integrity. At 3 days, an acute inflammatory response was observed with a rapid accumulation of cells resembling lymphocytes and

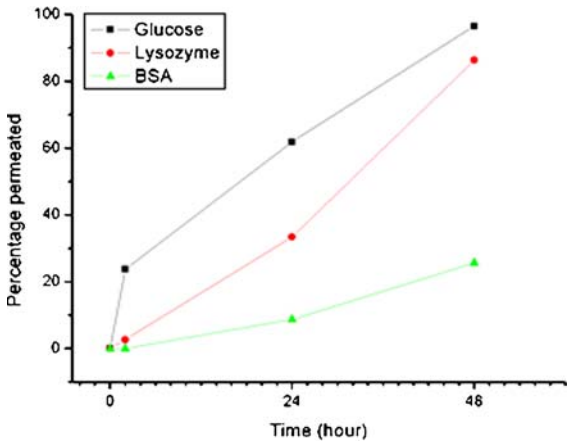

Fig. 2 Permeation of glucose (Mw 180 Da), lysozyme (Mw 14,600 Da) and BSA (Mw 66,200 Da) through the wall of a sealed chitosan nerve conduit at different time points. The conduits were permeable to all three substances. The permeation is expressed as percentage of substance initially loaded into the conduit and shows an Mw-dependent behavior. The data represents the mean of two independent experiments

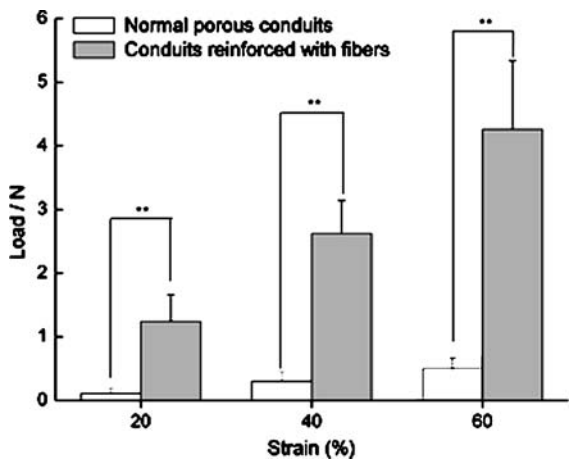

Fig. 3 The relationship between the load and strain during the compressive strength testing of the conduits. The load was significantly higher in the fiber-reinforced conduit group when compared to the unreinforced conduit group at each strain (**$P < 0.01$)

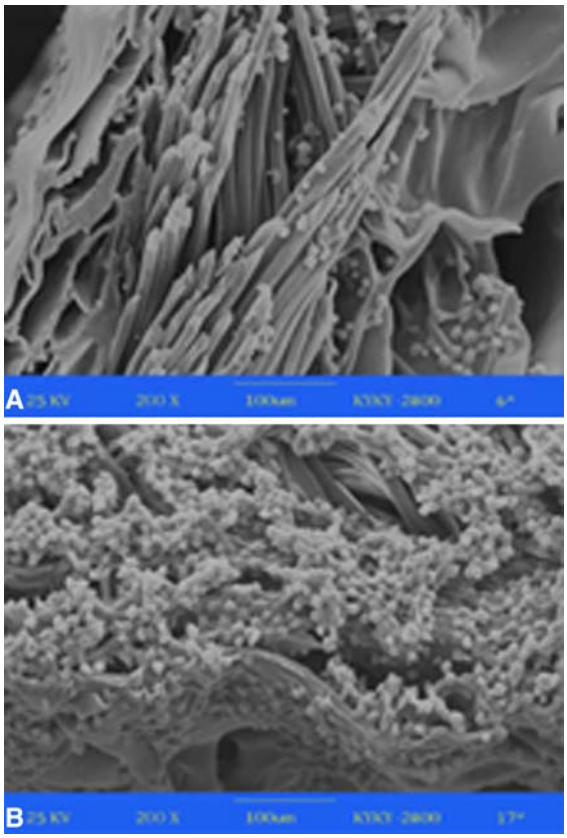

Fig. 4 Neuro-2a cell appearances after cultured on the reinforced chitosan conduits for 1 d (**A**) and 10 d (**B**). Cells growing on the conduit surface presented normal morphology and good proliferation from 1 d to 10 d, indicating that the conduits are cytocompatible

macrophages at the site between the conduits and the surrounding tissue. At 2 weeks, a loose capsule of connective tissue was present surrounding the implant. By 4 weeks, the fibrous tissue capsule became thinner and more compact and was accompanied by active neovascularization. At 8 weeks, the tissue capsule had become even thinner and obvious conduit/tissue integration was observed between the conduit exterior porous structure and the surrounding tissue (Fig. 5). This indicated that the conduits evoked only a mild tissue response and were compatible with the surrounding tissue.

Conclusions

We have developed a method to produce chitosan conduits with sufficient permeability and mechanical

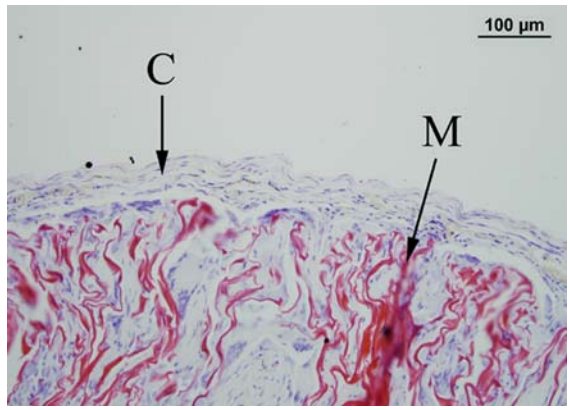

Fig. 5 Hematoxylin and eosin staining of an implanted conduit at 8 weeks. A dense uniform tissue capsule (C) was found surrounding the wall of the conduit. Tissue ingrowth into the exterior porous structure of the conduit was observed and the chitosan scaffold material (M) was seen to be well integrated with surrounding tissue

strength for nerve repair applications without any chemical additives or crosslinking. The conduits are also non-cytotoxic and tissue compatible, and therefore, represent a promising candidate conduit for peripheral nerve regeneration.

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