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Tailoring of chitosan scaffolds with heparin and γ-aminopropyltriethoxysilane for promoting peripheral nerve regeneration

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ABSTRACT

Chitosan has been well known for promoting peripheral nerve regeneration, however, its effect is still not as good as that of autografts. In this study, the feasibility of using negatively charged heparin and positively charged γ-aminopropyltriethoxysilane (APTE) treatment as biocompatible modification of lyophilized porous chitosanscaffolds was evaluated. The morphology of the prepared chitosan scaffolds as a function of treatment with different charged molecules showed no significant differences, while a skin-like surface was observed for the scaffolds modified with high APTE concentration and heparin. The quantitative and qualitative characterization of heparin and amino densities by Toluidine Blue O (TBO) and Acid Orange (AO) assays confirmed the successful immobilization of heparin and APTE on the chitosan scaffolds. The measurement of surface charge densities indicated that the scaffolds treated with APTE showed increased charge densities while heparin decreased the cationic charge density. Moreover, the fabricated charge processed chitosan scaffolds were stable after immersion in phosphate buffer saline for more than ten days. Further on, the chitosan scaffolds processed with 2 mg/mL heparin did facilitate the attachment, proliferation and maintain the biological function of Schwann cells *in vitro*. The study demonstrates that chitosan scaffolds treated with suitable heparin concentration provides an effective selection for biomaterials surface modification and shows great potential for the application in peripheral nerve regeneration.

1. Introduction

Currently, lesions of peripheral nerves caused by traumatic events or in the frame of tumor surgery seriously affects the patients' health and life. Generally, nerve tissue in peripheral nerve system (PNS) has only limited capability of self-regeneration, but for the damage of PNS with short gap, an end-to-end anastomosis *via* suturing is an effective method to bridge the nerve stump [1,2]. However, for the damage of PNS with long gap larger than 3 cm, complete recovery is still very difficult to realize [3]. As for now, the autologous graft is still the golden standard for repairing nerve injury with long gap, but it has disadvantages such as the limited availability of donor graft and the permanent surgery trauma at the donor site [4].

Until now, various biomaterials have been developed for the repair of peripheral nerve injury, including natural and synthetic polymers [5,6]. Chitosan, as a natural polysaccharide, has attracted more and more attention as it is non-toxicity, shows good biocompatibility, biodegradability, readily availability [2,7–9]. Both chitosan membranes and fibers had been found to possess excellent affinity for neuroglia cells affinity, suggesting a promising application for peripheral nerve repair [10]. Nie et al. [11] showed that the average regenerated myelin area, nerve conduction velocity and myelinated axon count could be well promoted by chitosan/gelatin nerve graft contaning nerve growth factor (NGF).

Though chitosan has been successfully applied for repairing nerve injury, it still does not satisfy the requirement of rapid nerve regeneration. The chemical or physical surface properties besides the bulk properties of biomaterials play an important role on the tissue or cell response. Among which the surface physics properties have attracted more and more attention in recent years, such as electric charges, topology and mechanics, *etc.* Electric charge is one of the most important influence factors in tissue regeneration, as

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it stimulates proliferation and differentiation of different cell types [12–14]. A photopolymerizable poly(L-lysine) (PLL) with positive charges was covalently incorporated into poly(ethylene glycol) diacrylate (PEGDA) hydrogels, and it was found that the adhesion and proliferation of neural progenitor cells was promoted on the PLL-grafted hydrogels [15]. Selective neuronal adhesion was regulated *via* charged groups by Liu et al. [16], the results showed that neurons did not form networks on the negative charged laminin grids, while they showed favourable adhesion on positively charged poly(ethylenimine) (PEI) films. In addition, a positively charged hydrogel was formed by copolymerizing oligo-(polyethylene glycol) fumarate (OPF), a biocompatible and biodegradable macromer, with [2-(methacryloyloxy) ethyl]-trimethylammonium chloride (MAETAC), the formed positively charged hydrogels could improve primary sensory rat neuron attachment and differentiation in a dose-dependent manner, and promote the appearance of myelinated structures in a coculture of dorsal root ganglion (DRG) and Schwann cells [17]. In our previous study, the chitosan scaffolds modified with positively charged APTE also promoted Schwann cell attachment and proliferation to some extent [18].

In the present study, the feasibility of using negatively charged heparin and positively charged APTE to modify lyophilized chitosan scaffolds was evaluated. The effects of charge modification on the scaffold performance including morphology, quantity and quality of heparin and amino groups, the variation of charge densities and degradation behavior were measured. Primary Schwann cells were seeded on the sterilized scaffolds and cultured statically *in vitro* for different periods of time, and the attachment and proliferation behaviors of the cells on negative and positive charge processed chitosan scaffolds were investigated and discussed.

2. Materials and methods

2.1. Materials

Chitosan powder (CS, Mw:2.8×10^4) was purchased from Nantong Xincheng Biochemical Company, Jiangsu, China, and the degree of deacetylation was >90%.γ-aminopropyltriethoxysilane (APTE), heparin (160 U/mg) from porcine intestinal mucosa human, forskolin, heregulin, Toluidine Blue O (TBO) and Acid Orange (AO) were all obtained from Sigma–Aldrich. 0.067 M phosphate buffer saline (PBS, pH 7), Dulbecco's modified eagle medium (DMEM), fetal bovine serum (FBS) and trypsin/EDTA solution were purchased from Hyclone Co. Ltd. Cell counting kit-8 (CCK-8) for Schwann cells viability and proliferation test were purchased from BD Biosciences, San Jose, CA. NGF kit, brain-derived neurotropic factor (BDNF) kit, and ciliary neurotrophic factor (CNTF) kit were obtained from Chemicon, Temecula, CA. All the other reagents used in the experiments were of the highest analytical purity (>99.9%).

2.2. Preparation of the charged chitosan scaffolds

Firstly, the porous CS scaffolds were prepared with the freeze-drying method according to our previous study [9]. Then, the prepared CS porous scaffolds were dried naturally and stored in a desiccator before use. For preparing the charged chitosan scaffolds, the scaffolds were immersed into a solution of APTE (in 99.8% anhydrous ethanol) and heparin solution (in PBS) with different concentrations (APTE: 2%, 5%, 10%, 15% and 20%, heparin: 2 mg/mL, 5 mg/mL, 10 mg/mL, 25 mg/mL and 50 mg/mL) at 37 °C for 5 h to generate silanized and heparinized chitosan scaffolds, respectively. Then the samples were washed thoroughly with ethanol or PBS to remove free APTE or heparin molecules. After that, the samples were dried in a 80 °C oven for 6 h and cut into wafers with 10 mm diameter before use. The names of APTE treated samples were denoted CSA2, CSA5, CSA10, CSA15 and CSA20, while heparin treated samples as CSH2, CSH5, CSH10, CSH25 and CSH50, respectively.

2.3. Morphological observation

The surface morphology of the charged chitosan scaffolds was observed using an optical microscopy (Leica, Germany) and a scanning electron microscopy (SEM, Hitachi S-3400 NII, Japan).

2.4. Qualitative and quantitative characterization of amino groups

The amino groups on different charged chitosan scaffolds were quantitatively characterized by AO staining as described by Li et al. [19]. The absorbancy (OD) was recorded by a microplate reader (Bio-Tek Inc., USA) at 485 nm. The density of amino groups was calculated according to a calibration curve. The calibration curve was prepared as following: The OD values of AO with a serious of known concentrations in NaOH (pH 12) solution were measured at 485 nm.

For qualitative characterization of amino groups, the charged samples were immersed in 500 μmol/L AO-HCl (pH3) solution in water in a 500 μL well. After shaking for 1 h at 37 °C, the samples were rinsed three times with HCl (pH 3), and then the samples were observed using an optical microscope.

2.5. Qualitative and quantitative characterization of heparin

The heparin on different chitosan scaffolds were quantitatively characterized by TBO staining method as described in the literature [20]. The OD value was obtained using a microplate reader at 530 nm. The amount of immobilized heparin was calculated from a calibration curve. The calibration curve was prepared as following: the TBO with a known volume was added to a heparin solution with a series of known concentration and shaken at 37 °C for 4 h. Then, the Hep/TBO complex was precipitated. The mixture was centrifuged (3500 rpm, 10 min). Subsequently, the precipitate was rinsed and dissolved in a mixture of ethanol and NaOH. The absorbance at 530 nm was measured by microplate reader.

For qualitative characterization of heparin, the samples were soaked in the solution of 0.04 wt% TBO in aqueous 0.01 M HC1/0.2 wt% NaC1. Then, the samples were gently shaken for 1 h at 37 °C. After washing five times with dH_2O (totally 15 min), the samples were observed by an optical microscope.

2.6. Measurement of the surface charges

The surface charges of the prepared chitosan scaffolds were measured using a EST111 Static Charge Meter (EST Electro-Static Test, Co. Ltd., China.) as following: the chitosan scaffolds with the diameter of 10 mm were placed in the measurement chamber, the data were record when the reading was stable. And the mean value of the surface charges was calculated from at least three individual measurements.

2.7. Release of APTE and heparin from chitosan scaffolds

The *in vitro* release of APTE and heparin from charged chitosan scaffolds was evaluated by AO and TBO tests after immersing the samples in PBS for different periods. In brief, the charged samples were immersed in a 24-well culture plate containing 2 mL/well of release medium (PBS), and the culture plate was placed on a horizontal shaker with a speed of 100 rpm at 37 °C for 1, 3, 7 and 14 days, respectively. PBS was changed every three days. Then the soaked