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Effect of silanization on chitosan porous scaffolds for peripheral nerve regeneration

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ABSTRACT

The aim of this study was to evaluate the feasibility of using 3-aminopropyltriethoxysilane (APTE) silanization treatment for modification and biocompatibility of lyophilized chitosan porous scaffolds. The process is beneficial for biomaterial development due to its low toxicity and simplicity. The silanization treatment with low APTE concentration showed no significant influence on the morphology of chitosan scaffolds, while a skin-like surface was observed for the silanized scaffolds treated with high APTE concentration. The porosity and surface amino densities were increased after silanization whereas the swelling ratio was reduced, and the degradation ratio in PBS and anti-acid degradation properties of the silanized chitosan scaffolds were significantly improved. The *in vitro* Schwann cells culture demonstrated that the silanized scaffolds with 8% APTE could obviously facilitate the attachment and proliferation of Schwann cells, indicating great potential for the application in peripheral nerve regeneration.

1. Introduction

The peripheral nerve injury (PNI) is a serious health problem for the trauma patients caused by disease, war or traffic accident. Generally, an end-to-end anastomosis method can be used to bridge the PNI with small gaps, however, it is still difficult to completely cure the long nerve gaps (Ichihara et al., 2009). The autologous nerve graft is the best choice for repairing nerve injuries, but its source is limited and the wound at the donor site is permanent.

In recent years, a variety of biomaterials for better recovery of nerve functions have been developed (Hsu, Chan, Chiang, Chen, & Jiang, 2011; Kuo & Chang, 2013; Pettersson et al., 2011; Runge et al., 2010; Xu, Yan, & Li, 2011), including natural and synthetic polymers. Chitosan (CS), as a natural polysaccharide, has attracted more and more attention due to its good biocompatibility, biodegradability, non-toxicity, readily availability and unique physicochemical properties (Busilacchi, Gigante, Mattioli-Belmonte, Manzotti, & Muzzarelli, 2013; Qu, Lin, Zhang, Xue, & Zhang, 2013; Wang et al., 2005; Wlaszczuk, Pietrucha-Dutczak, Marcol, Jedrzejowska-Szypulka, & Lewin-Kowalik, 2011; Yang et al., 2011; Yuan, Zhang, Yang, Wang, & Gu, 2004), and have been used in various areas of tissue engineering (Muzzarelli, 2009). Yuan et al. studied the interaction of Schwann cells with chitosan scaffolds and fibers, and found that both the chitosan scaffolds and fibers had excellent neuroglial cell affinity, suggesting a promising application of chitosan for nerve regeneration (Yuan et al., 2004). Wei et al. fabricated the collagen–chitosan scaffold and further immobilized RGD sequences to mimic the bio-functional peripheral nerve, they found that this scaffold could promote rapid regeneration of injured sciatic nerve in rats (Xiao et al., 2013). The chitosan/gelatin nerve graft was also developed for delivering Schwann cells and nerve growth factor (NGF) to explore the feasibility of improving sciatic nerve regeneration, the results showed that the nerve conduction velocity, average regenerated myelin area, and myelinated axon count were all promoted (Nie et al., 2013). Xu et al. developed a more complex conduit composed of poly-DL-lactide, chondroitin sulfate and chitosan, and further immobilized NGF on the conduit with carbodiimide, the conduit was found to enhance rapid functional recovery of the disrupted nerve without connective tissues from ingrowth, indicating the conduit would be useful material to repair peripheral nerve damage (Xu et al., 2011). In addition, the differentiation of induced pluripotent stem (iPS) cells into neuron-like cells was found to be accelerated by the chitin–chitosan–gelatin scaffolds (Kuo & Lin, 2013). As mentioned above, despite the wide range of chitosan related biomaterials for nerve repair available, it is still unacceptable about the functional recovery of seriously injured nerve due to the scar formation, which may inhibit the regeneration process. Therefore, it is necessary to further improve the properties of chitosan for promoting nerve regeneration *via* various physicochemical methods.

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Generally, the biomaterials surface plays an important role on cell response to the implants, thus surface modification of the biomaterials will be beneficial for improving the biocompatibility. Silanization is a convenient method that can be used to cover the biomaterials surface through the covalent binding of organofunctional alkoxysilane molecules and surface hydroxyl groups. Aminosilanes display potentially useful because they could be used to directly or indirectly (through a linker) immobilize biomolecules on biomaterials surface (Baggi, Boschi, Caleffi, Martignoni, & Venanzi, 1990), which are important for subsequential cell or tissue responses. Various silane agents have been applied to modify the inorganic and organic biomaterials surface in terms of different purposes, including glass (Halliwell & Cass, 2001), dental ceramics (Matinlinna & Vallittu, 2007), titanium (Li et al., 2011) and natural fiber/polymer composites (Salon, Abdelmouleh, Boufi, Belgacem, & Gandini, 2005). 3-Aminopropyltriethoxysilane (APTE) is one of the most widely used silane agents for fabricating the amine-terminated scaffolds (Kim, 2011; Lapin & Chabal, 2009). Yoshioka, Tsuru, Hayakawa, and Osaka (2003) reported that the alginic acid layers immobilized on gamma-APTE-grafted stainless-steel could obviously inhibit platelet adhesion and improve blood compatibility. Li et al. (2011) grafted heparin/fibronectin complex on titanium surface *via* APTE silanization, and showed that the modified surface could simultaneously promote endothelialization and inhibit thrombosis formation. In most cases, APTE is used to silanize inorganic biomaterials surface in order to provide amino reactive sites for biomolecules grafting. Though there are some reports referring to the silanization of different polymers (Gan, Yang, & Yang, 2009; Salon et al., 2005), the effect of silanization treatment on the natural biomaterials is still unknown, especially for the biological response.

In the present study, the feasibility of using APTE to silanize the lyophilized chitosan scaffolds was evaluated. The effects of silanization on the scaffolds performances including morphology, porosity, swelling rate and degradation behavior were measured. The primary Schwann cells were seeded on the sterilized scaffolds and cultured statically *in vitro* for different periods. The attachment and proliferation behaviors of the cells on silanized chitosan scaffolds were investigated.

2. Materials and methods

2.1. Materials

Chitosan powder (Mw: 2.8×10^4) with a degree of deacetylation of 92.3% was purchased from Nantong Xincheng Biochemical Company, Jiangsu, China. 0.067 M phosphate buffer saline (PBS, pH 7) and Dulbecco's modified eagle medium (DMEM) were purchased from Hyclone Co., Ltd. Forskolin, heregulin, 3-aminopropyltriethoxysilane (APTE), Toluidine blue O (TBO) and acid orange II (AO II) were all purchased from Sigma–Aldrich. A 1 wt% acetic acid solution was prepared by diluting 1 mL acetic acid in 100 mL deionized water (dH_2O). All the other reagents used in the experiments were of the highest analytical purity (>99.9%).

2.2. Preparation of the silanized chitosan scaffolds

The preparation of the silanized chitosan scaffolds is systematically shown in Fig. 1. Firstly, the chitosan scaffolds were prepared using the following method: the chitosan powder was dissolved in a 1 wt% acetic acid aqueous solution to form a 1 wt% chitosan solution at room temperature, the solution was stationary for 2 h to remove the trapped air bubbles. Subsequently, the chitosan solution was poured into a cell culture dish and lyophilized in a freeze dryer at −50 °C for 24 h to form porous chitosan scaffolds. After the drying stage, the formed chitosan scaffolds were immersed in a 0.1 M NaOH aqueous solution for 24 h to neutralize the residual acetic acid. Then the scaffolds were rinsed with dH_2O for 8∼10 times to remove the residual alkali. Finally, the scaffolds were dried and stored in dehumidifying device before use. Secondly, the silanized chitosan scaffolds were prepared using silanization method as follows: the chitosan scaffolds above were silanized by immersing into a 2%, 5% and 8% v/v solution of the APTE in anhydrous ethanol for 5 h, respectively. The silanized chitosan scaffolds by APTE was separately denoted CSA-2%, CSA-5% and CSA-8%, then the samples were washed thoroughly with ethanol in order to remove the physisorbed APTE molecules. After that, the samples were kept in a 120 °C oven for 6 h to enhance the binding of APTES with the chitosan scaffolds.

2.3. Morphology of the prepared chitosan scaffolds

The surface morphology of the prepared chitosan scaffolds was observed using an optical microscopy (OM, Leica, Germany) and a scanning electron microscopy (SEM, Hitachi S-3400 NII, Japan), respectively. For SEM observation, briefly, the test samples were fixed to an aluminum stage using a double-sided adhesive tape, and then coated with gold to a thickness of 50 nm using a gold sputter coater machine. Finally, the coated samples were observed by SEM under the vacuum degree of 1.33×10^{-4} Pa.

2.4. FTIR

The infrared absorption spectra of the pristine chitosan, CSA-2%, CSA-5%, and CSA-8% were obtained from a FTIR spectrometer (Nicolet5700, Madison, WI) in transmission mode. For each spectrum obtained, a total of 64 scans were accumulated at 4 cm^{-1} resolution. Scanning was conducted in the range from 400 to 4500 cm^{-1}.

2.5. Porosity rate

The porosity rate of the chitosan scaffolds before and after silanization was measured by a liquid displacement method reported by Yoshioka et al. (2003). Briefly, the scaffold was immersed in anhydrous ethanol with a known volume (V_0), and then a series of vacuum-release cycles were performed to force the liquid into the pores of the scaffold. Thereafter, the volume of the liquid-perfused scaffold and liquid was recorded as V_1. Subsequently, the liquid-perfused scaffold was taken out, and the remaining liquid volume was recorded as V_2. Finally, the porosity rate of the scaffolds was calculated using the following equation:

$$\frac{V_0 - V_2}{V_1 - V_2} \times 100\%$$

2.6. Measurement of amino groups

The density of the amino groups was determined by AO experiment as following: the chitosan samples were immersed in 500 μmol/L AO-hydrochloric acid (HCl) (pH 3) solution dissolved in water. After shaking for at least 1 h at 37 °C, the samples were rinsed three times with pH 3 HCl solution. Then, the samples were immersed into pH 12 NaOH solution and shaken for 15 min at room temperature to dissolve the adsorbed AO. Finally, 150 μL of desorbed AO supernatant was added to a 96-well plate, and the optical density (OD) was recorded with a microplate reader (Bio-Tek Inc.,