Carbohydrate Polymers 84 (2011) 788–793

Contents lists available at ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Controlled release of chondroitinase ABC in chitosan-based scaffolds and PDLLA microspheres

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ARTICLE INFO

Article history:
Received 27 September 2009
Received in revised form 10 February 2010
Accepted 19 March 2010
Available online 2 April 2010

Keywords:
Spinal cord injury
Chondroitin sulfate proteoglycans
Chondroitinase ABC
Chitosan
Nerve conduits
Poly(D,L-lactic acid)
Microspheres

ABSTRACT

Spinal cord injury (SCI) can trigger inhibitory signal cascades that promote the expression of chondroitin sulfate proteoglycans (CSPGs), which are the main structures in scar tissue. The digestion of CSPGs by chondroitinase ABC (ChABC) can promote axonal re-growth after SCI. However, ChABC cannot effectively digest CSPGs because it is unstable; therefore, stable ChABC must be released in a controlled manner in the repair of SCIs. Two methods of maintaining ChABC stability and bioactivity were examined. They were the immobilization of ChABC on nerve conduits (NCs) and the encapsulation of ChABC in poly(D,L-lactic acid) (PDLLA) microspheres.

Nerve conduits with variously sized pores were fabricated from chitosan and gelatin. The pore diameters of chitosan NCs were 100–160 μm, and those of chitosan/gelatin NCs were 20–40 μm. The ChABC in NCs was immobilized by ionic or covalent bonding. The experimental results reveal that immobilizing ChABC in NCs markedly improved its stability. The activity of ChABC that was immobilized in chitosan NCs by ionic bonding was 0.07 U/mg; 48% of this activity was retained at 48 h after immobilization. PDLLA microspheres, fabricated by the double emulsion method, were applied as carriers in the controlled release of ChABC. Stabilizers, including nanogold (10 nm), polylysine (Mw: 500–2000) and polylysine (Mw: 20,000–30,000), were added to microspheres to maintain the activity of ChABC. Polylysine (Mw: 500–2000) stabilized ChABC most effectively. The ChABC activity was 0.0162 U/ml after seven days of release. Experimental results indicate that ChABC activity can be preserved during release by immobilizing ChABC in chitosan NCs and encapsulating ChABC in PDLLA microspheres using an appropriate stabilizer.

1. Introduction

Injury to a spinal cord can induce inhibitory signal cascades that promote the formation of scar tissue. Such scars comprise a dense collagen IV meshwork, which functions as a binding matrix for numerous extracellular matrix components, inhibitory molecules, including proteoglycans and semaphorins, and growth-promoting factors (Klapka & Muller, 2006). Chondroitin sulfate proteoglycans (CSPGs), the principal inhibitors of neurite outgrowth, are up-regulated by spinal cord injury (SCI) (Busch & Silver, 2007). The CSPGs consist of a core protein with several covalently linked glycosaminoglycan (GAG) side chains. The GAGs is a key limited factor to block axon growth. Additionally, chondroitinase ABC (ChABC) digests GAG side chains, promoting functional recovery following SCI (Bradbury et al., 2002; Huang et al., 2006). However, a significant challenge to be met is that ChABC is too unstable to maintain its activity *in vivo* when ChABC is employed in SCI.

In 2002, Bradbury et al. injected Wistar rats with ChABC following SCI (Bradbury et al., 2002). In their experiments, a silastic tube was inserted intrathecally, such that it was just rostral to the lesion site, and externalized to deliver bolus injections of highly pure, protease-free ChABC. However, ChABC must be injected every other day because it is unstable. Such treatment is both inconvenient and painful. To control the continuous release of ChABC, Ikegami et al. utilized an osmotic pump for ChABC infusion to digest the CSPG in the injured spinal cord at one to two weeks after SCI (Ikegami et al., 2005). Huang et al. inserted a catheter for ChABC infusion following T8 completely spinal cord transaction (Huang et al., 2006). This

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doi:10.1016/j.carbpol.2010.03.034

study aims to immobilize ChABC on NCs and encapsulate ChABC in microspheres to control the release of ChABC.

Chitosan and PDLLA were adopted to fabricate NCs and microspheres because of their biocompatibility and biodegradability (Huang, Khor, & Lim, 2004; Tsuji, 2005). Furthermore, chitosan has excellent nerve repair potential (Gong et al., 2000). A porous chitosan NC was previously formed by a lyophilizing and wire-heating process (Huang, Huang, Huang, & Liu, 2005). PDLLA has been fabricated in various forms for biomedical applications. For example, electrospun fiber mats are used as carriers for topical and/or transdermal delivery, and nanocomposites are used to release drugs (Chuysinuan, Chimnoi, Techasakul, & Supaphol, 2009; Dagnon et al., 2009). In this work, chitosan NCs and PDLLA microspheres are applied as carriers to release ChABC over a defined period to promote nerve regeneration. The effects of stabilizers, including nanogold and polylysine with various molecular weights, were also examined.

2. Materials and methods

All reagents were purchased from Sigma Aldrich (Oakville, ON, CA), unless otherwise stated. Poly(D,L-lactic acid) (PDLLA) was purchased from Bio Invigor (Taiwan). Poly (vinyl alcohol) (PVA) that was 80% hydrolyzed with a molecular weight of 6000 D was purchased from Wako (Japan). The MicroBCA Protein Assay Kit was purchased from Pierce (Rockford, IL, USA). Deionized water was obtained from Milli-RO 10 Plus and Milli-Q UF Plus (Bedford, MA, USA) water purification units at 18 MV resistance.

2.1. Preparation of chitosan or chitosan/gelatin scaffold

For preparing chitosan scaffold, the chitosan (DDA: 82.5 ± 1.15%, Mw 645,000)/acetic acid solution (2%, w/v) was injected into a mold and then frozen at −20, for 24 h. The acetic acid was then sublimated using temperature-controlled lyophilizers (VirTis, NY, USA). After lyophilization, the scaffolds were washed by 1:1 0.1 M NaOH–MeOH and 1:1 MeOH–water in order to neutralize the acid, and then dried again by lyophilizers. For preparing chitosan/gelatin scaffold, the chitosan scaffold already fabricated was soaked in gelatin solution (5%) for 1 min, cross-linked by glutaraldehyde (1%) for 30 s and then dried again by lyophilizers. Finally, the scaffolds were stored in a dry environment until use.

2.2. Immobilization of ChABC on chitosan-based scaffolds

The ChABC in the scaffolds were immobilized using two methods. The first is as follows. Prior to immobilization, the chitosan or chitosan/gelatin scaffold was washed with ChABC buffer solution (250 mM Tris HCl and 300 mM sodium acetate, at pH 8.0). Then 1 mg of washed scaffold was soaked in 100 μl ChABC solution (10 U/ml) at 4 °C overnight in a closed container to prevent evaporation. The scaffold was then washed again using ChABC buffer solution to remove un-immobilized ChABC. Next, 0.2 ml ChABC buffer solution that contained glutaraldehyde (GTA) (0.1–0.2%) was added to the scaffold at 4 °C for one hour, and the excess GTA was then removed.

In the second method, 1 ml 1%(v/v) GTA solution was added to 1 mg scaffold for three hours at room temperature, and then 100 μl ChABC (10 U/ml) was added. Changing the order in which these two chemicals, ChABC and GTA are added, changes the chemical bonds.

2.3. Preparation of microspheres

The PDLLA microspheres were prepared by standard double emulsion/solvent evaporation. Briefly, 0.1 g PDLLA was dissolved in 2 ml dichloromethane (CH_2Cl_2), and 200 μl of aqueous bovine serum albumin (BSA) or ChABC (20 U/ml) was then added. The mixture was stirred for 1.0 min at 4000 rpm and then added to 20 ml of a previously prepared aqueous 1 wt.% PVA solution, which was stirred for another 1 min at 2000 rpm. Finally, the entire volume was poured into a flask with ice and stirred for three hours (Caframo Stirrer Type RZR, speed setting 3.0) to evaporate CH_2Cl_2. Following all of the solvent had evaporated, the microspheres were washed three times with centrifugation (12,000 rpm) in distilled water. After the final centrifugation, the wet microspheres were freeze-dried and stored in a dry environment under aseptic conditions prior to use.

To fabricate microspheres that contained stabilizers, including nanogold (10 nm) and polylysine (Mw: 500–2000 or 20,000–30,000), to maintain ChABC activity, a ChABC/stabilizer solution was used instead of ChABC solution alone in the preparation process. In total, 10 U ChABC was added to 200 μl polylysine (5 mg/ml) or nanogold to prepare the ChABC/stabilizer solution.

2.4. Bioactivity of ChABC

The bioactivity of ChABC that was released from chitosan-based scaffolds or PDLLA microspheres was evaluated *in vitro* by determining its capacity to digest chondroitine sulfate (CS) to produce unsaturated disaccharide. The amount of unsaturated disaccharide was determined by ultraviolet (UV) absorbance at 232 nm using a Hitachi U-3010 UV–Vis photometer using a calibration curve for describing the absorbance–concentration relationship (Pojasek, Shriver, Kiley, Venkataraman, & Sasisekharan, 2001). The ChABC bioactivity was calculated as

$$\text{Units/ml} = \frac{(\Delta A_{232\,\text{nm}}/\text{ min test})(1.0)(\text{df})}{(E_{\text{mM}})(0.10)(0.8)}$$

df: factor of dilution; E_{mM}: mini-molar absorption coefficient (Ernst et al., 1996; Pojasek, Shriver, Hu, & Sasisekharan, 2000); $\Delta A_{232\,\text{nm}}$/min test: (absorption difference at 232 nm after reaction)/reaction time.

2.5. Determination of amount of ChABC released in vitro

The efficiency of the release of ChABC was determined from its bioactivity. The chitosan-based scaffold (0.001 g) was put into buffer solution containing CS (2%, 500 μl) for 5 min at 37 °C. Then, 100 μl buffer was mixed with 900 μl KCl (pH 1.8) solution at 90 °C to terminate the reaction. The bioactivity of ChABC was calculated using the absorption of supernatants at 232 nm. To release ChABC from PDLLA microspheres, 1.00 ml Tris–HCl sodium acetate solution was added to 0.05 g ChABC/PDLLA microspheres. At various times, 80 μl supernatant was mixed with 20 μl CS solution at 37 °C for 5 min; the reaction was then stopped at 90 °C. The absorption of the supernatant was quantified using ultraviolet (UV) absorbance at 232 nm.

The efficiency of release from PDLLA microspheres *in vitro* was determined using BSA instead of ChABC. The experimental processes are the same as those described above, although the detection method is different. The amount of BSA that was released in the supernatant was determined using BCA protein assay kits.

3. Results

3.1. Chitosan-based scaffolds

Fig. 1 presents the morphological changes of the chitosan scaffold when NaOH/H_2O or NaOH/methanol was used as a neutralizer. The scaffold that was neutralized by NaOH/H_2O shrank more by volume (50%) than did that neutralized by NaOH/methanol (30%). A comparison of scaffolds reveals that the pore diameter was greater in the NaOH/methanol group. These experimental results