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Structure, morphology and properties of genipin-crosslinked carboxymethylchitosan porous membranes

Anderson Fiamingo, Sergio Paulo Campana-Filho*

Instituto de Química de São Carlos, Universidade de São Paulo, Av. Trabalhador são-carlense, 400, CEP, 13566-590 São Carlos, SP, Brazil

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ABSTRACT

Highly porous genipin cross-linked membranes of carboxymethylchitosan exhibiting different crosslinking degree (3% < $\overline{CrD}$ < 18%) were produced by using different concentrations of genipin and carboxymethylchitosan possessing high, medium or low molecular weight. The membranes were able to adsorb high amounts of PBS and presented high ultimate tensile strength and elongation-at-break the lower the crosslinking degree and the higher the molecular of the parent carboxymethylchitosan. Particularly, the membrane prepared from high molecular weight carboxymethylchitosan displayed higher swelling ratio (17.5 g/g), ultimate tensile strength (≥300 kPa) and elongation-at-break (≥65%). The susceptibility to lysozyme degradation depends only on the crosslinking degree of the membranes, the degradation rate being faster the lower the crosslinking degree. The preparation of lightly genipin cross-linked carboxymethylchitosan membranes displaying appropriated properties to fulfill specific applications as biomaterials is envisaged by using high molecular weight carboxymethylchitosan.

1. Introduction

Chitosan, a linear β(1 → 4)-linked copolymer of 2-amino-2-deoxy-D-glucopyranose (GlcN) and 2-acetamido-2-deoxy-D-glucopyranose (GlcNAc) units in which the former units predominate, is usually produced by treating chitin with aqueous alkali (Roberts, 1992). In chitin, a polysaccharide widely found in nature, the GlcNAc units are predominant and the hydrolysis of its acetamido groups to result in GlcN units converts chitin into chitosan. Such a conversion is commonly named as chitin deacetylation, the resulting polymer being named as chitosan if it is formed predominantly by GlcN units and is soluble in dilute aqueous solution. Thus, chitin and chitosan differ in terms of chemical composition and with respect to solubility as chitin is insoluble in aqueous media regardless of its pH as well as in most organic solvents.

Owing to its biodegradability, biocompatibility and low toxicity, chitosan has been proposed for a number of applications in dentistry, pharmacy, medicine and tissue engineering (Domard, 2011; Ravi Kumar, 2000; Rinaudo, 2006). However, the limited solubility of chitosan, restricted to acidic aqueous media, precludes many of its potential applications and several chemical modifications have been proposed to improve the polymer solubility as well as to develop new functionalities and properties (Chen & Park, 2003; de Abreu & Campana, 2009; Ledung, Milas, Rinaudo, & Desbrieres, 1994; Roberts, 1992). In this sense, the reactive sites in the chitosan are its hydroxyl and amino groups and the main reactions include carboxymethylation, quaternization, sulfation, PEGylation, alkylation and acylation, among others (Rinaudo, 2006; Roberts, 1992).

Carboxymethylchitosan (CMCh), an anionic derivative of chitosan usually produced by reacting chitosan with monocholoroacetic acid in alkaline medium, exhibits improved water solubility (de Abreu & Campana, 2009; Upadhyaya, Singh, Agarwal, & Tewari, 2013), gel-forming capacity (Chen, Wu, Mi, Lin, Yu, & Sung, 2004; Lin, Liang, Chung, Chen, & Sung, 2005) and enhanced biological activities, such as biocompatibility (Chen, Wang, Liu, & Park, 2002), biodegradability, low immunogenicity (Fu, Han, Dong, Yang, Lv, & Liu, 2011), antimicrobial activity (Kim, Thomas, Lee, & Park, 2003; Liu, Wang, Zhuang, Wu, Yang, & Zeng, 2012; Liu, Guan, Yang, Li, & De Yao, 2001; Rabea, Badawy, Stevens, Smagghe, & Steurbaut, 2003) and antioxidant activity (Sun, Zhou, Xie, & Mao, 2007; Zhao, Huang, Hu, Mao, & Mei, 2011). Indeed, such interesting properties and biological activities of CMCh have stimulated the studies aiming to investigate the potential use of this chitosan derivative in medicine and tissue engineering (Bao, Chen, Wang, Wang, Liu, & Sun, 2014; Lopes et al., 2010; Oliveira et al., 2009; Upadhyaya, Singh, Agarwal, & Tewari, 2013). Thus, as carboxymethylchitosan shows structural similarity to heparin, a sulfated glycoaminoglycan which stabilizes the family of

* Corresponding author. Tel.: +55 16 33739929; fax: +55 16 33739952.
E-mail address: scampana@iqsc.usp.br (S.P. Campana-Filho).

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heparin-binding growth factors (HBGF) and stimulates the cellular growth, Lopes et al. (2010) proposed the use of viscous aqueous solution of CMCh containing keratinocyte growth factor (KGF) to reduce the severity of postoperative pericardial adhesions in large animal model. Indeed, such study has shown a synergistic effect due to the association KGF/CMCh which resulted in significant decrease of post-surgical adhesions. However, as viscous solutions and physically crosslinked gels may be fully washed-out within few days by the organism turnover, the potential applications of CMCh as a polymeric scaffold in tissue engineering call for physical and chemical stability, *i.e.*, its degradation rate must occur in the due time. Additionally, CMCh scaffolds must be able to swell in biological fluids, to allow the cell colonization and the exchange of nutrients and metabolites, and to withstand mechanical stress.

In recent years, self-sustainable CMCh-based films, hydrogels and membranes have been developed by carrying out the polymer crosslinking using different crosslinking agents such as, for instance, glutaraldehyde (Liu, Huang, Peng, Ding, & Li, 2007), epichlorohydrin (Demarchi et al., 2014), 1-ethyl-3(3-dimethylaminopropyl) carbodiimide hydrochloride (Lu et al., 2007; Lu, Sheng, Wang, Wei, Gong, & Zhang, 2009; Reves, Bumgardner, & Haggard, 2013), poly(*N*-vinyl-2-pyrrolidone) (Agustina Aldana, Gonzalez, Strumia & Martinelli, 2012), oxidized starch (Baran, Mano & Reis, 2004), oxidized dextran (Hoffmann, Seitz, Mencke, Kokott & Ziegler, 2009), oxidized sugars (Li, Liu & Yao, 2002) and genipin (Bao, Chen, Wang, Wang, Liu & Sun, 2014; Muzzarelli, 2009; Reves, Bumgardner & Haggard, 2013).

Aiming to modulate the main properties of a crosslinked polymer network, it is important to choose the proper physical, ionic and/or covalent crosslinking agent and to control the crosslinking extent. However, as most synthetic crosslinking agents exhibits high cytotoxicity and low biocompatibility, natural compounds should be preferred to develop crosslinked polymer networks to be applied in tissue engineering. In this sense genipin, a natural compound isolated from the fruits of *Gardenia jasminoides* Ellis (Fujikawa, Yokota, Koga, & Kumada, 1987) which exhibits very low cytotoxicity as compared to glutaraldehyde (Mi, Tan, Liang, & Sung, 2002; Sung, Chen, Huang, Hsu, & Chang, 2000), has been proposed as crosslinking agent to produce biocompatible, noncytotoxic polymer-based scaffolds potentially useful for tissue engineering and drug-delivery applications (Agustina Aldana et al., 2012; Li et al., 2015; Li, Nan, Shi, & Chen, 2012).

The aim of this study is to develop porous genipin-crosslinked membranes prepared from carboxymethylchitosan and to investigate the effects of the polymer molecular weight and the extent of crosslinking on the morphology, swelling ratio, mechanical properties and susceptibility to *in vitro* lysozyme degradation of the CMCh membranes.

2. Experimental

2.1. USAD chitosan

Beta-chitin extracted from squid pens (*Doryteuthis spp.*) was submitted to the ultrasound-assisted deacetylation process, namely USAD process (Delezuk, Cardoso, Domard, & Campana, 2011), to produce extensive deacetylated, high molecular weight chitosan. Thus beta-chitin was suspended in aqueous 40% NaOH (w/w), the suspension (1/10 w/v) was poured in a double walled glass reactor (θ_{int} = 3.5 cm) coupled to a thermostat (60 °C ± 1 °C) and then it was submitted to ultrasound irradiation for 50 min. A Hielscher Sonifier UP400S ultrasonic device (ν = 24 kHz) coupled to the sonotrode (θ = 22 mm) was employed and pulsed irradiation at constant ultrasound power (200 W) was adjusted. The above described process was repeated three times consecutively to result in the USAD chitosan named as sample Ch0.

2.2. Carboxymethylchitosan

USAD chitosan Ch0 (10.0 g) was suspended in 100 mL of water/isopropanol (1/4 v/v) containing 13.5 g of NaOH and the suspension was stirred for 1 h at 30 °C. Following, monochloroacetic acid (15 g) dissolved in isopropanol (20 mL) was slowly added to the suspension and the reaction was allowed to proceed for 4 h at 30 °C (Chen & Park, 2003; Liu, Guan, Yang, Li, & De Yao, 2001). The reaction was interrupted by addition of ethanol (200 mL), the product was isolated by filtration, and then it was extensively washed with ethanol and dried at room temperature. The resulting carboxymethylchitosan, named as sample CMCh0, was purified by dissolving it in aqueous 0.1 mol/L NaCl, filtering the resulting solution to remove insoluble particles and gels, and adding ethanol to provoke the precipitation of the polyelectrolyte, which was extensively washed with ethanol and dried at room temperature.

Two depolymerized carboxymethylchitosan samples, namely CMCh1 and CMCh3, were produced by submitting 500 mL of an aqueous solution of CMCh0 (10 g/L) contained in a 1 L glass beaker (θ_{int} = 10 cm) to ultrasound irradiation (pulsed irradiation at 200 W and temperature at 60 °C) during 1 h and 3 h, respectively. After the ultrasound-induced depolymerization, samples CMCh1 and CMCh3 were recovered upon neutralization with aqueous NaOH, filtration, washing and drying, and then they were purified as described above.

2.3. Characterization of chitosan and carboxymethylchitosan

2.3.1. Infrared spectroscopy

A film was prepared by dissolving the sample (1.0 mg) in 1.0 mL of 0.1 mol/L HCl aqueous solution, pouring the polymer solution in a silicon wafer and allowing the evaporated the solvent at 30 °C. The infrared spectrum of the sample in film form was acquired by using the FTIR BOMEM MB102 spectrophotometer in the range between 4000 and 400 cm^{-1} by accumulation of at least 64 scans, with a resolution of 4 cm^{-1}.

2.3.2. NMR spectroscopy

The ^{13}C and ^{1}H NMR spectra were acquired at 80 °C by using a Bruker AVANCE III spectrometer (υ = 400 MHz), 2,2-dimethyl-2-silapentane-5-sulfonic acid (DSS) being added to the solutions of the samples as an external reference. The composite pulse sequence "ZGCPPR" was used to suppress the signals of hydrogens from water. The concentration of polymer solutions were 10 mg/mL in the D_2O/HCl (100/1 (v/v)) and 100 mg/mL in the D_2O to acquire the ^{1}H and ^{13}C NMR spectra, respectively.

The average degrees of acetylation ($\overline{DA}$) and substitution ($\overline{DS}$) of the polymers were determined from the respective ^{1}H and ^{13}C NMR spectra according to the literature (Chen & Park, 2003; Hirai, Odani, & Nakajima, 1991; Rinaudo, Dung, Gey, & Milas, 1992; Signini & Campana, 1999).

2.3.3. Capillary viscosimetry

The intrinsic viscosity ([η]) was determined by using the AVS-360 (SCHOTT) viscometer coupled to the automatic burette (TITRONIC universal-SCHOTT) as described elsewhere (Delezuk, Cardoso, Domard, & Campana, 2011). Chitosan was dissolved in aqueous solution of 0.3 mol/L acetic acid/0.2 mol/L sodium acetate (pH = 4.5) while carboxymethylchitosan was dissolved in 0.1 mol/L NaCl aqueous solution and the intrinsic viscosities were determined from the corresponding curves of the reduced viscosity