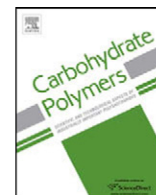
Carbohydrate Polymers 91 (2013) 92–99

Contents lists available at SciVerse ScienceDirect

Carbohydrate Polymers

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



Sulfated chitosan as tear substitute with no antimicrobial activity

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ARTICLE INFO

Article history:
Received 12 June 2012
Received in revised form 2 August 2012
Accepted 3 August 2012
Available online 10 August 2012

Keywords:
Sulfated chitosan
FTIR
NMR
Viscosity
Tear substitute
Antimicrobial activity

ABSTRACT

Chitosan of high molar mass and with 82% deacetylation was sulfated using two procedures and characterized. In the first method sample chitosan-S1 was produced using chlorosulfonic acid as the sulfating agent and *N*,*N*-dimethylformamide as the medium, and in the second method (chitosan-S2) formic acid was also used. The degrees of sulfation were 0.87 (chitosan-S1) and 0.67 (chitosan-S2). FTIR spectra showed bands at 1230, 800 and 580 cm^{-1}, attributed to sulfation. Moisture content followed the order: chitosan-S-0.87 > chitosan-S-0.67 > chitosan. Chain depolymerization was verified by GPC. Aqueous solutions showed pseudoplastic behavior and the viscosity at a concentration of 0.3% (w/v) was higher than that of healthy human tears (close to 3 mPa s at shear rate 130 s^{-1}). Substitutions in the C2—NH and in C6—OH groups were verified by NMR. Antimicrobial activity against *Staphylococcus aureus* and *Pseudomonas aeruginosa* was not observed. Considering that chitosan-S-0.67 had a higher solubility, less chain depolymerization, higher yield and better thermal stability in comparison with chitosan-S-0.87, the derivative with DS 0.67 offered the greatest potential for use in formulations of tear substitutes.
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1. Introduction

Tear replacement by topical application of artificial tears is the most widely used therapy for reduce pain in patient with dry eye syndrome (DES) (Calonge, 2001). The syndrome is a multifactorial disease of the ocular surface and tear film and has been recently recognized as a public health problem due to its prevalence (5–30% of population) and impact on life quality (Miljanovic, Dana, Sullivan, & Schaumberg, 2007; Yao, Davidson, Durairaj, & Gelston, 2011). DES is characterized by a deficiency in the quantity or quality of tears, an unstable tear film, and ocular surface damage (Miljanovic et al., 2007). Ocular pain, foreign body sensation, redness, and photophobia are the bothersome symptoms (Yao et al., 2011). If left untreated, this syndrome can lead to serious complications, including loss of vision.

Several commercial brands of artificial tears or tear substitutes are available (Calonge, 2001; Vibhute, Kawtikwar, Kshirsagar, & Sakarkar, 2010). They are based on synthetic or natural polymers. Poly(vinyl alcohol), poly(acrylic acid), poly(vinyl pyrrolidone) and polyethyleneglycol are some of the synthetic polymers used. The natural materials are polysaccharides such as hyaluronic acid, methylcellulose, carboxymethylcellulose, hydroxyethylcellulose and hydroxypropyl-guar (Gifford, Evans, & Morris, 2006; Ketelson, Davis, & Meadows, 2009). Lipidic emulsions are also commercialized.

Chitosan is the linear and partly acetylated (1-4)-2-amino-2-deoxy-β-D-glucan (Muzzarelli et al., 2012). Chitosan exhibits well-known properties, such as biodegradability, nontoxicity, biocompatibility, mucoadhesiveness, and antibacterial activity (Tang et al., 2010). Due to these favorable properties, chitosan is an important material in ophthalmological research (Basaran & Yazan, 2012; Felt et al., 1999; Fuente et al., 2010; Yang, Wang, Gu, & Zhang, 2008).

The use of soluble derivatives of chitosan as tear substitutes was suggested more than a decade ago by Argueso et al. (1998). However, chitosan is water insoluble in neutral medium, as in the case of the proposed application. A cationic water soluble, chitosan hydrochloride, was tested, due to its good wetting properties as well as the antibacterial effect (Felt, Carrel, Baehni, Buri, & Gurny, 2000). Unfortunately, the chitosan derivative precipitates in the tear film (Ludwig, 2005).

Based on their mucoadhesive capacity, charged polymers are better for DES treatment in comparison to non-ionic materials (Ludwig, 2005). In fact, polymer solutions and emulsions used in artificial tear formulations, such as hyaluronate, acrylic acid, and carboxymethylcellulose, are mostly anionic at physiological pH (Calonge, 2001; Fuente et al., 2010).

Sulfated chitosan is a water-soluble anionic chitosan derivative, with antiviral (Nishimura et al., 1998), anticoagulant (Vikhoreva et al., 2005), antimicrobial (Huang, Du, Zheng, Liu, & Fan, 2004), and osteogenic activity (Zhang, Peschel, Helm, Groth, & Fischer, 2011;

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0144-8617/$ – see front matter © 2012 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.carbpol.2012.08.011

Zhou et al., 2009). This derivative also blocks human malignant melanoma cell adhesion (Wang, Huang, Wei, & Zeng, 2010), and shows anti-obesity effect by the promotion of anti-adipogenesis inhibition (Karadeniz, Karagozlu, Pyun, & Kim, 2011). In addition, sulfated chitosan has low cytotoxicity (Nishimura et al., 1998).

In this study, chitosan was sulfated by two different methods and the derivatives were characterized. The ideal tear substitutes for DES must be water soluble, free from preservatives, protect against microbial contamination (Asbell, 2006), and have viscosity higher than that of natural human tears and adequate osmolality (Vibhute et al., 2010). Those properties will be measured in order to verify the potential application of sulfated chitosan as artificial tear.

2. Experimental

2.1. Materials

N,N-Dimethylformamide (DMF) and chlorosulfonic acid (CSA) were obtained from Vetec. High molecular weight chitosan (HMWCh, $M_v = 4.6 \times 10^5$ g/mol) with a deacetylation degree (DD) of 82% was purchased from Polymar Ciência e Nutrição S/A (Brazil). Other chemicals were all of analytical grade and used as received. The dialysis membrane was purchased from Sigma–Aldrich and had an approximate molar mass cut-off of 12,400 g/mol. The phosphate buffer saline (pH 7.4) was 0.2 mol/L in phosphate and 0.15 mol/L in NaCl.

2.2. Sulfation of chitosan

2.2.1. Method I

Sulfated chitosan (chitosan-S1) was obtained according to the method of Gamzazade et al. (1997) with minor modifications. The sulfating complex was obtained by dropwise addition of 4.5 mL $HClSO_3$ with stirring to 30 mL DMF previous cooled at 4 °C. The reaction mixture was stirred without cooling until the solution reached room temperature. Chitosan (2.0 g) was added to 30 mL DMF and stirred for 12 h at room temperature. The excess of solvent was eliminated by filtration to give a solvated chitosan. The solvated polysaccharide was added to the sulfating complex and the reaction was run at room temperature for 5 h with stirring. The final mixture was neutralized by 20% (m/v) NaOH and precipitated with methanol in an ice bath. The precipitated was dissolved in water and the solution dialyzed against distilled water for 3 days with two water changes per day. The solid chitosan-S1 was recovered by lyophilization.

2.2.2. Method II

Sulfated chitosan (chitosan-S2) was prepared using a method similar to that reported by Zhou et al. (2009) with modifications. The sulfating complex and the solvated chitosan were obtained as in Method 1 but modifying the amount of reagents. In the sulfating complex the amounts used were: $HClSO_3$ (5.0 mL instead of 4.5 mL) and DMF (50 mL instead of 30 mL). For the solvation, 2.5 g chitosan (in contrast to 2.0 in Method 1) were added to the same volume of DMF. Also, in Method 2 an additional stage was included in the process, that is, the mixture of the solvated polysaccharide with 50 mL of DMF plus 2 mL of formic acid. The sulfating complex was then added and the reaction was run at room temperature for 3 h with stirring. The product was precipitated with 700 mL EtOH, filtered under vacuum, washed with EtOH and dried with hot air. The precipitate was dissolved in water and the pH adjusted to 7 with NaOH 20% (w/v). The undissolved material was removed by filtration, and the solution dialyzed against water for 3 days. After lyophilization the chitosan-S2 was obtained.

2.3. Characterization of sulfated chitosan samples

Depending on the reaction conditions, the sulfo group can be incorporated in C-2 (NH_2 group) giving 2-*N*-sulfated chitosan, in C-6 (CH_2OH group) giving 6-*O*-sulfated chitosan, or in C-3 (OH group) to give 3-*O*-sulfated (Rakhmanova et al., 2009). The sulfo group can also be introduced in more than one position, such as in C-2 and in C-6, with the synthesis of 2-*N*, 6-*O*-sulfated chitosan (Zhou et al., 2009). The total degree of sulfation represents the sum of the degree of sulfation in all groups (Fig. 1). The total DS was ascertained from the sulfur content (%S) determined by elemental analysis using a Perkin-Elmer CHNS 2400 analyzer, and the calculation provided by Eq. (1) (adapted from Melo, Feitosa, Freitas, & de Paula, 2004):

$$DS = \frac{169 \times (\%S)}{3200 - 102 \times (\%S)} \tag{1}$$

where 169 g/mol is the average molar mass of the chitosan repeating unit taking into account the DD of 82%, 3200 is 100 times the atomic mass of sulfur, and 102 g/mol is the molar mass of SO_3Na minus 1 related to the H lost during sulfation.

Thermogravimetric analysis (TGA) of the samples in a platinum crucible was carried out on a TA Instruments Q50 analyzer with a heating rate of 10 °C/min over the temperature range of 25–800 °C. The air flow rate was maintained at 60 mL/min and the initial sample weight was 10 mg.

FTIR spectra were recorded with KBr pellets on an FT-IR Shimadzu 8300 spectrophotometer in the range of 4000–400 cm^{-1}.

The elution volumes were determined by gel permeation chromatography (GPC) using a Shimadzu LC-10AD chromatograph with an RID-6A refractive index detector at room temperature. The analysis was performed with a PolySep linear column (7.8 mm × 300 mm), flow rate of 0.5 mL/min, polysaccharide solution concentration of 0.1% (w/v), water as the solvent and 0.1 mol/L $NaNO_3$ as the eluent. The sample volume was 50 μL.

Rheological studies of chitosan derivatives were performed in phosphate buffer saline (pH 7.4) aqueous solutions (0.3%, w/v), on an AR550 rheometer (TA Instruments) at 36 °C with a 40 mm cone plate sensor. The effect of shear rate on the solution viscosity was evaluated.

2D (^1H–^{13}C HSQC) spectra of 3% (w/v) solutions in D_2O at 343 K were recorded on a Fourier transform Bruker Avance DRX 500 spectrometer. An inverse multinuclear gradient probe-head equipped with z-shielded gradient coils and a Unix Silicon Graphics workstation was used. Sodium 2,2-dimethylsilapentane-5-sulfonate (DSS) was used as the internal standard (0.00 ppm for ^1H).

The osmolality of aqueous solutions at a 0.3% (w/v) concentration in phosphate buffer saline (PBS) was measure in a Wescor® vapor pressure osmometer (model Vapro® 5520) at room temperature. The calibration was carried out with NaCl standard solutions at concentrations of 100, 290 and 1000 mOsm/kg. The volume of the samples and standard solutions was 10 μL.

2.4. Antibacterial activity

The effect of chitosan sulfate on bacterial growth was evaluated by measuring the growth of the experimental and control cultures by optical density at 600 nm. The human pathogen Gram-positive *Staphylococcus aureus* ATCC 6538 and Gram-negative *Pseudomonas aeruginosa* ATCC 9027 were chosen for test. The bacteria were grown in Tryptone Glucose Yeast Extract Broth (TGE) pH 7.2 at 37 °C for 24 h under shaking (160 rpm), and the cell densities of both cultures adjusted to 0.1 at 600, which corresponding to 10^7–10^8 Colony Forming Unit (CFU) mL^{-1}. For the assay, 500 μL of each culture were inoculated in tubes containing 5 mL of sterilized (0.22 μm) chitosan sulfate solutions, prepared in phosphate buffered saline pH 7.2, at 0.003%, 0.03% and 0.3% (m/v)

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