Sulfated chitosan as tear substitute with no antimicrobial activity

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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 reducing pain in patient with dry eye syndrome (DES) (Calonge, 2001). The syndrome is a multifactorial disease of the ocular health problem 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, Shirsagar, & Sakarkar, 2010). They are based on synthetic or natural polymers. Poly(vinyl alcohol), poly(acrylic acid), poly(vinyl pyrrolidone) and polyethylene glycol 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 carboxy methylcellulose, 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;
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, Karagözlu, 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

\[
\text{N,N-Dimethylformamide (DMF) and chlorosulfonic acid (CSA) were obtained from Vetec. High molecular weight chitosan (HMWCh, } M_0 = 4.6 \times 10^6 \text{g/mol) with a deacetylation degree (DD) of 82}\% \text{ was purchased from Polymar Ciência e Nutrição S/A (Brazil). Other chemicals were all of analytical grade and used as received. The dialysis membrane was purchased from Sigma-Aldrich and had an approximate molar mass cut-off of 12,400 g/mol. The phosphate buffer saline (pH 7.4) was 0.2 mol/L in phosphate and 0.15 mol/L in NaCl.}
\]

2.2. Sulfation of chitosan

2.2.1. Method I

Sulfated chitosan (chitosan-S1) was obtained according to the method of Gamazzade et al. (1997) with minor modifications. The sulfating complex was obtained by dropwise addition of 4.5 ml HCISO₃ with stirring to 30 ml DMF previously 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 I but modifying the amount of reagents. In the sulfating complex the amounts used were: HCISO₃ (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 I) 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 sulfogroup can be incorporated in C-2 (NH₂ group) giving 2-N-sulfated chitosan, in C-6 (CH₂OH group) giving 6-O-sulfated chitosan, or in C-3 (OH group) to give 3-O-sulfated (Rakhmanova et al., 2009). The sulfogroup 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 sulfate content (%) 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):

\[
\text{DS} = \frac{169 \times (\%)}{3200 - 102 \times (\%)}
\]

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 SO₃Na 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 NaNO₃ 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–13C HSQC) spectra of 3% (w/v) solutions in D₂O 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 0.3% (w/v) concentration in phosphate buffer saline (PBS) was measured 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, 250 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 (TGY) 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 10⁷–10⁸ 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)
concentrations. The tubes were incubated at 37 °C under agitation (160 rpm) for 18 h. Controls were prepared in the same way without chitosan sulfate. The assays were done in triplicate.

3. Results and discussion

3.1. Total degree of sulfation (DS)

The sulfur content was 10.59 and 9.03% for chitosan-S1 and chitosan-S2, respectively. The total DS, determined from equation 1, was higher for chitosan-S1 (0.87) than for chitosan-S2 (0.67). Table 1 compares the %S of the sulfates obtained from different chitosan samples (varying the M₀ and DD) and through reactions carried out under various conditions (sulfating agent, temperature, time and medium). The sulfated chitosan can be prepared in a homogeneous or heterogeneous way (Gamzazade et al., 1997; Zhang et al., 2011). The molar ratio of sulfating agent to chitosan units varied from 1.9 to 16. The low ratios (1.9–4) were used for low molecular weight chitosan (LMWCh, M₀ ≈ 103–104 g/mol).

Sulfation of HMWCh (M ≈ 105 g/mol) was performed with molar ratios ranging from 4 to 16. The range of 4–9 is the most commonly employed.

The reaction temperature did not exceed 85 °C. Sulfation at room temperature as well as at a lower temperature (0–4 °C) is also reported, the latter being performed to avoid a high degree of depolymerization due to the presence of a strong sulfuric acid solution. The reactions were performed for 1–26 h.

The %S observed in the sulfation of various chitosan samples under different conditions varied from 8.0 to 16.4 (Table 1). Sulfating with chlorosulfonic acid provided higher sulfur content when the medium contained formic acid (FA) or dichloroacetic acid (DCA). The sulfur percentages for chitosan-S1 and chitosan-S2 were 10.59 and 9.03%, respectively. The value observed for chitosan-S1, denoted hereafter as chitosan-S-0.87 is very close to that reported by Zhang et al. (2011) (sample CHS15), who used a lower molar ratio and shorter reaction time, but a higher temperature. In comparison with the sulfated chitosan prepared by Zhou et al. (2009) (sample 26SCS) the %S for chitosan-S2 denoted hereafter as chitosan-S-0.67

![Chemical structures of chitosan modified with different sulfate groups](image)

**Fig. 1.** Chemical structures of chitosan modified with different sulfate groups: (I) 6-O-sulfated chitosan (C-6); (II) 3-O-sulfated chitosan (C-3); (III) 2-N-sulfated chitosan (C-2); and (IV) 2-N, 6-O-sulfated chitosan (C-2 and C-6) (Zhou et al., 2009; Rakhmanova et al., 2009).

Table 1

<table>
<thead>
<tr>
<th>Sample</th>
<th>Chitosan</th>
<th>Medium</th>
<th>Sulfating agent</th>
<th>Molar ratio</th>
<th>T °C/h</th>
<th>%S</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>25SCS</td>
<td>&gt;90</td>
<td>2.5 × 10⁵</td>
<td>H₂SO₄, H₂SO₃</td>
<td>70/60</td>
<td>50/16</td>
<td>8.0</td>
<td>Zhou et al. (2009)</td>
</tr>
<tr>
<td>CHS18</td>
<td>&gt;96</td>
<td>2.2 × 10⁴</td>
<td>DMF, formamide</td>
<td>70/60</td>
<td>50/16</td>
<td>14.1</td>
<td>Zhang et al. (2011)</td>
</tr>
<tr>
<td>CHS20</td>
<td>&gt;96</td>
<td>2.5 × 10⁵</td>
<td>DMF, formic acid</td>
<td>70/60</td>
<td>50/16</td>
<td>14.1</td>
<td>Zhang et al. (2011)</td>
</tr>
<tr>
<td>SCH8</td>
<td>&gt;90</td>
<td>2.5 × 10⁵</td>
<td>H₂SO₃, DMF</td>
<td>70/60</td>
<td>50/16</td>
<td>14.1</td>
<td>Zhang et al. (2011)</td>
</tr>
<tr>
<td>(N,O)SC</td>
<td>&gt;90</td>
<td>2.5 × 10⁵</td>
<td>H₂SO₃, DMF</td>
<td>70/60</td>
<td>50/16</td>
<td>14.1</td>
<td>Zhang et al. (2011)</td>
</tr>
<tr>
<td>CHS15</td>
<td>&gt;96</td>
<td>2.5 × 10⁵</td>
<td>H₂SO₃, DMF</td>
<td>70/60</td>
<td>50/16</td>
<td>14.1</td>
<td>Zhang et al. (2011)</td>
</tr>
<tr>
<td>HCTS3</td>
<td>87</td>
<td>2.5 × 10⁵</td>
<td>H₂SO₃, DMF</td>
<td>70/60</td>
<td>50/16</td>
<td>14.1</td>
<td>Zhang et al. (2011)</td>
</tr>
<tr>
<td>HCTS10</td>
<td>87</td>
<td>2.5 × 10⁵</td>
<td>H₂SO₃, DMF</td>
<td>70/60</td>
<td>50/16</td>
<td>14.1</td>
<td>Zhang et al. (2011)</td>
</tr>
<tr>
<td>Chitosan-S1</td>
<td>82</td>
<td>2.5 × 10⁵</td>
<td>H₂SO₃, DMF</td>
<td>70/60</td>
<td>50/16</td>
<td>14.1</td>
<td>Zhang et al. (2011)</td>
</tr>
<tr>
<td>Chitosan-S2</td>
<td>82</td>
<td>2.5 × 10⁵</td>
<td>H₂SO₃, DMF</td>
<td>70/60</td>
<td>50/16</td>
<td>14.1</td>
<td>Zhang et al. (2011)</td>
</tr>
</tbody>
</table>

DD: degree of deacetylation; FM: formamide; FA: formic acid; DMF: dimethylformamide; DCA: dichloroacetic acid; Oleum: SO₃ in sulfuric acid; NI: not informed; RT: room temperature.

Identification of samples according to the authors.

Medium: solvents and/or reagents used to activate chitosan.

Molar ratio: mol sulfating agent per mol GlcN units.

T °C/h: reaction temperature in °C and reaction duration in hours.
is lower, which is explained by the lower molar ratio and temperature used in this study. The addition of sufficient formic acid to dissolve all of the chitosan (10 ml of FA to 1 g of chitosan), a condition used by Zhang et al. (2011), leads to a much higher %S (15.7) when compared with the value for chitosan-S-0.67 (2 ml of FA per 2.5 g of chitosan). Moreover, Zhang et al. (2011) used a higher molar ratio and time of reaction.

The total DS, determined from Eq. (1), was higher for chitosan-S-0.87 than for chitosan-S-0.67. The sulfation yields were 0.22 g/g for chitosan-S-0.87 and 0.48 g/g for chitosan-S-0.67. Previously reported yields obtained in other studies are much higher, for instance, 1.7 g/g (Xing et al., 2005), 1.1 g/g (Vikhoreva et al., 2005), and 0.83 g/g (Xing et al., 2005). Loss of material during dialysis is the most probable cause of the low yield in the present study.

3.2. Thermogravimetric analysis

The thermogravimetric curves (TGA and DTG) for the chitosan and derivatives are shown in Fig. 2 and the parameters obtained from the curves in Table 2. In all samples one water loss and two decomposition events can be seen, occurring at different temperatures. The higher hydrophilicity resulting from the sulfation can be explained by the trend in the moisture content (chitosan-S-0.87 > chitosan-S-0.67 > chitosan). The sulfated sample with higher DS (0.87) presented lower temperatures ($T_i$ and $T_{max}$) and lower thermal stability than chitosan-S-0.67. The greater amount of sulfated material residue at 800 °C is attributed mainly to the counter ion (Na$^+$) of the sulfate group. The difference between the chitosan-S-0.87 and chitosan-S-0.67 residues is in the range of experimental variation.

3.3. FTIR spectroscopy

Fig. 3 shows the FTIR spectra for the chitosan and derivatives and Table 3 shows the band assignments. On comparing the spectra some changes due to sulfation can be seen. New bands appear in the ranges of 1261–1266, 1226–1228, 1065–1074, 1001–1005, 941, 805–813 and 580–582 cm$^{-1}$. The signals at 1261–1266, 1226–1228 cm$^{-1}$ represent asymmetric stretching of SO$_2$ and the band of the symmetric vibration appears at close to 1070 cm$^{-1}$. The band at around 800 cm$^{-1}$ is attributed to the C—O—S stretching vibration, and SO$_2$ deformation appears at 580–582 cm$^{-1}$ (Vikhoreva et al., 2005). A small band at 941 cm$^{-1}$ was present in the spectra of 6-O-sulfated chitosan and not in that of 2-N-sulfated (Zhou et al., 2009). Thus, it was attributed to the C—O—S vibration. A band close to 1000 cm$^{-1}$ has been noted in spectra obtained for chitosan sulfate prepared by various authors (Huang et al., 2004; Vikhoreva et al., 2005; Xing et al., 2005; Zhou et al., 2009) independent of whether the substitution is in the carbon or nitrogen. This band can be assigned to a sulfate group.

Some wavelength shifts are noted. The bands at 2920 and 2884 cm$^{-1}$ in the chitosan spectrum (stretching vibration of CH,
CH₂ and CH₃ shift to 2960 and 2900 cm⁻¹. Shifts to lower wave-lengths occur for the band at 1654 cm⁻¹. The signal related to NH and NH₂ deformation shifts from 1562 cm⁻¹ to 1530 cm⁻¹ and becomes weaker after sulfation. The band at 1376 cm⁻¹ shifts slightly to 1382–1383 cm⁻¹ and the vibration of CH₃ (1424 cm⁻¹) decreases in intensity. The sulfate groups are expected to change the vibration modes of methyl, methylene, methin, and amino groups (Zhang et al., 2011).

Some indication of the position of sulfation can be obtained from the FTIR spectra in the region of O–H and N–H stretching vibration (3200–3500 cm⁻¹). In the chitosan spectra, a broad band with a maximum at 3410 and shoulder at 3250 cm⁻¹ confirm the presence of OH and NH groups, respectively. Zhou et al. (2009) have reported that the spectra of chitosan sulfated only in the amino group present a band at 3360 cm⁻¹ ascribed to νOH. When the sulfate group was inserted in C6–OH the authors recorded two bands, one at 3500 cm⁻¹ (νOH) and the other at 3210 cm⁻¹ (νNH). The difference in the region of 3200 cm⁻¹ between ChS1 and ChS2 suggests that less NH₂ groups remained in ChS1 and consequently that the degree of substitution in the amino group was greater than in chitosan-S-0.87 than in chitosan-S-0.87.

3.4. Gel permeation chromatography (GPC)

The absolute value of the molar mass of sulfated products cannot be determined by GPC without applying an appropriate technique such as multiangle laser light scattering (MALLS) detection. Therefore, the discussion on molar mass will be comparative and based only on elution volumes and the negative charge of sulfated chitosan. If no chain depolymerization occurs, the more sulfated samples would be expected to elute at a lower volume than the less sulfated polymer, due to the molar mass increase and to the higher repulsion between the groups of the anionic polyelectrolyte, which have a greater charge.

The increase in molar mass is due to the introduction of the -SO₃Na group. In the chitosan-S-0.87 and chitosan-S-0.67, if no depolymerization occurs, the molar masses will increase 54 and 41%, respectively. The elution of the chitosan-S-0.87 sample yielded a higher volume (9.84 mL) than that of the chitosan-S-0.67 (9.50 mL) sample. Therefore, it can be assumed that chain depolymerization occurred during the preparation of chitosan-S-0.87. The low yields obtained in the chitosan-S-0.87 (0.22 g/g) and chitosan-S-0.67 (0.48 g/g) preparation indicate that the polymer with a molecular weight of less than 1.24 × 10⁴ (membrane cut-off point) was lost during the dialysis. The chitosan has a high molecular weight (4.6 × 10⁵). Chain depolymerization occurs during chitosan-S-0.87 and also chitosan-S-0.67 synthesis, but is higher for chitosan-S-0.87.

Chain depolymerization during sulfation of LMWCh was reported by Vikhoreva et al. (2005). The authors did not compare the Mₚ values but rather the degree of polymerization (DP), which is a more appropriate parameter. The DP falls from 274 to 75 after sulfation (sample SCH8, Table 1). Calculations based on the Mₚ data reported by Zhou et al. (2009) showed that the DP decreased from 400 to 170 for LMWCh (sample 26SCS). Chain depolymerization was lower for HMWCh (DP from 1500 to 1300) sample 25CS). A significant decrease in the DP during the sulfation of HMWCh has been reported by Xing et al. (2005). In samples identified as HCTS3 and HCTS6, in Table 1, the chitosan DP (4700) falls to 324 and 196, respectively. The yield was inversely proportional to the depolymerization degree: HCTS3, yield = 1.73 g/g and HCTS6, yield = 0.83 g/g.

The chain depolymerization during the preparation of chitosan-S-0.87 can be explained by the higher molar ratio of sulfating agent and longer reaction time applied. The lower yield is in agreement with the higher degree of depolymerization.

3.5. Rheology

Formulations for tear substitutes based on polysaccharides are prepared in concentrations varying from 0.1 to 0.7% (w/v) (Vibhute et al., 2010). Rheological analysis of the chitosan-S-0.87 and chitosan-S-0.67 aqueous solution was performed at 0.3% (w/v) in PBS at 36 °C at shear rates of 15–1000 s⁻¹ (Fig. 4). The samples showed a non-Newtonian behavior, indicating a pseudoplastic fluid (shear-thinning), similar to natural human tears (Gouveia & Tiffany, 2005). The rheology of tears includes two processes: blinking and inter-blinking. In a closed eye, the ocular shear rate is zero. During inter-blinking periods the ocular shear rate is 0.03 s⁻¹ which rises to 4250–28500 s⁻¹ during blinking (Kumar & Himmelestein, 1995). The viscosity has to be high at low shear rates and low at high shear rates, showing a typical pseudoplastic behavior.

Human tears have a viscosity of around 3 mPa s (Ludwig, 2005). Gouveia and Tiffany (2005) observed viscosity values of 2.33 mPa s and 0.97 mPa s at shear rates of 0.0175 s⁻¹ and 128.5 s⁻¹, respectively. Zero-shear viscosity values of 4.4, 7.1 and 8.3 mPa s have been determined for natural tears (Tiffany, 1991). As expected with natural systems, there is a normal variation between individuals.

Artificial tears must have higher viscosity than natural tears. They are delivered intermittently and the contact time with the...
ocular surface must be increased, in order to increase the duration of the effect. The residence time is proportional to the viscosity (Vico, Quereda, Benítez-del Castillo, Fernández, & García-Sánchez, 2005). Values in the range of 6–12 mPa s are considered as the viscosities for ideal tear substitutes for use in cases of dry eye syndrome (Vibhute et al., 2010; unfortunately, the shear rate was not specified by the authors). The value should not be higher than 30 mPa s because this leads to an unpleasant feeling for the patients (Oechsner & Keipert, 1999).

Solutions of sulfated chitosan at a concentration of 0.3% (w/v) and shear rates of 15 and 130 s⁻¹ showed viscosities of 13.5 and 2.73 mPa s for chitosan-S-0.87 and 12.4 and 3.25 mPa s for chitosan-S-0.67, respectively. At the same shear rates, human natural tears presented viscosities of 1.25 and 0.97 mPa s, respectively (Gouveia & Tiffany, 2005). Therefore, the viscosities of chitosan-S-0.87 and chitosan-S-0.67 are higher than that of natural human tears, in the range of 6–12 mPa s, and are lower than 30 mPa s. The chitosan derivatives thus show appropriate rheological properties for use in tear substitute formulations.

3.6. Choice of derivative

The water solubility behaviors of the chitosan derivatives vary considerably. The chitosan-S-0.67 sample maintains its solubility in water during storage at room temperature for up to 1 year. The other derivative (chitosan-S-0.87) is water-soluble when freshly prepared, but over time it becomes partially insoluble, probably due to aggregation. To avoid future problems related to solubility the study was continued only with chitosan-S-0.67. Besides the solubility behavior, this derivative was chosen for the following reasons: higher yield, lower chain depolymerization, and higher thermal stability.

3.7. Osmolality

The osmolarity values for the 0.3% (w/v) solution of chitosan-S-0.67 in water and in PBS were 139 ± 2 and 481 ± 2 mOsm/kg, respectively. The ideal value is considered to be 302 ± 6.3 mOsm/L (Vibhute et al., 2010). The cause of the high osmolality of chitosan sulfate in PBS is the NaCl concentration at 0.15 mol/L which gives around 300 mOsm/kg. Taking into account that 1 mol of NaCl dissociates into 2 moles of solute particles (1 osmole of Na⁺ and 1 osmole of Cl⁻), it is easy to fit the osmolality to the ideal range by varying the sodium chloride concentration. In fact, the commercial formulation must also contain K⁺, Ca²⁺, Mg²⁺, and Zn²⁺ (Vibhute et al., 2010). The most important results is the low osmolarity of chitosan derivative, which allows its adjustment to the ideal value by addition of the required ions and this will be the focus of a future study.

3.8. Nuclear magnetic resonance

The sulfation reaction of chitosan can produce derivatives such as 6-O-sulfate, 3,6-O-sulfate, N-sulfate (sulfate at amino group at C-2) and N,6-O-sulfate (Gamzazade et al., 1997; Holme & Perlin, 1997; Zhang et al., 2010, 2011). The HSQC spectrum for the chitosan-S-0.67 sample is shown in Fig. 5. The 1H⁻¹H HSQC correlation for C-6/H-6 shows a low intense signal at δ60.6/4.0, 3.7 due to CH-6 without the sulfate group and new signals at δ67.0/4.3 and δ66.6/4.1 assigned to sulfated C-6 (Gamzazade et al., 1997; Holme & Perlin, 1997; Zhang et al., 2010, 2011). The C-2 amino carbon of chitosan-S-0.67 shows three different correlations on the HSQC spectrum in the region of δ55.7 and δ56.7. This split may be correlated to different environments of the C-2 amino carbon as the sulfate group is inserted at this carbon (δ55.7/3.8 (C-2 amino); 56.0/3.4 due to acetylamino carbon and 56.4/3.15 due to sulfated amino carbon). Based on the HSQC spectrum the sulfation of chitosan-S-0.67 has sulfate groups at C-6 and the amino group of C-2, with greater substitution in C-6. This result is in agreement with those of the FTIR study.

3.9. Antibacterial activity

The solution of chitosan-S-0.67 was not able to inhibit the growth of S. aureus and P. aeruginosa even at the highest tested concentration, which was 0.3% (w/v). The optical densities of both cultures after 18 h of incubation in presence of chitosan sulfate did not differ from their controls. The absorbances for S. aureus and P. aeruginosa at the end of the exponential phase in presence of chitosan sulfate were respectively 1.34 ± 0.05 and 1.20 ± 0.01, while for their controls were 1.11 ± 0.12 and 1.10 ± 0.03, respectively. The inhibition of S. aureus growth was reported by Huang et al. (2004). The effect on the P. aeruginosa was not found in literature.
The antibacterial activity of chitosan depends on its degree of deacetylation (DD), its molecular weight, its concentration in solution, the pH of the medium and microorganism species (Rabea, Badawy, Stevens, Smaghe, & Steurbaut, 2003). Chitosan with a higher DD tends to have a higher antimicrobial activity.

The correlation between bacterial activity and molecular weight is subject of controversy (Kong, Chen, Xing, & Park, 2010). In general, high molecular weight chitosan acts mainly on the outer surface of the bacteria. Low molecular weight chitosan (MW ≤ 5000 g/mol) can permeate into cell and block the transcription of RNA (Benhabiles et al., 2012) and shows better growth inhibition activity toward bacteria (Kumar, Varadaran, Gowda, & Tharanathan, 2005).

Few studies are available concerning the antibacterial activity of sulfated chitosan. Low sulfated polysaccharide (%S0.63) obtained from shrimp chitosan (DD 69%) was found to inhibit the growth of Shigella dysenteriae, Aeromonas hydrophila, Salmonella typhimurium, and Bacillus cereus. High sulfur content (%S 13.03) adversely influenced its antibacterial effect (Chen, Liu, & Tsai, 1998). The activity against Escherichia coli and S. aureus was other reported study. Chitosan sulfate (DD 85.2 and DS 0.87) showed inhibition against Gram-positive S. aureus, but no effect against Gram-negative E. coli (Huang et al., 2004).

The lack of antibacterial activity of chitosan-S-0.67 is probably caused by the impossibility of cell penetration due to its high MW. The amount of sulfated groups is in excess compared to the amount needed to chelate trace metals. This somehow cancels the effect of inhibition in the toxins production. The bacterial activity of chitosan sulfate against S. aureus (Chen et al., 1998) can be explained by lower MW. The increase from 82 to 85 in DD is not enough to explain the difference in activity. The difference in DS is not a plausible reason, since chitosan-S-0.67 presented the lower value. A much lower DS and/or molecular weight is probably needed to give activity to chitosan-S-0.67.

4. Conclusions

Two chitosan sulfates with sulfation degrees of less than 1.0 (chitosan-S-0.87 and chitosan-S-0.67) and low reaction yields were obtained. Substitutions in C2-NOH and in C6-OH were suggested by FTIR and verified by NMR spectroscopy. Chain depolymerization was verified and this was higher during chitosan-S-0.87 than chitosan-S-0.67 synthesis. The moisture content followed the order: ChS1 > ChS2 > Ch. The chitosan-S-0.67 sample presented higher thermal stability than chitosan-S-0.87. Solutions at 0.3% (w/v) in phosphate buffer saline, pH 7.4, of both derivatives showed pseudoplasic behavior, a requirement of polymers to be used as tear substitutes. The viscosity values are higher than those of natural human tears, and close to 3 mPas at shear rate 130 s⁻¹. The solubility of chitosan-S-0.87 decreases with time of storage, a behavior not presented by chitosan-S-0.67. Substitutions in C2-NOH and in C6-OH were verified by NMR for chitosan-S-0.67. The osmolarity of the aqueous solution of this derivative was lower than the ideal value for artificial tear formulations, but it can be adjusted by the addition of required salts, such as NaCl and KCl. Considering the solubility behavior, lower chain depolymerization, higher yield, higher thermal stability the chitosan-S-0.67 presented high potential for use in tear substitute formulations even with no antibacterial activity.

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