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Preparation and characterization of chitosans carrying aldehyde functions generated by nitrogen oxides

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ABSTRACT

In this work, aldehyde-functionalized chitosan is produced by the reaction of chitosan with nitrogen oxides generated *in situ* from a HNO_3/H_3PO_4–$NaNO_2$ mixture. This method is more advantageous than the existing approaches, since the depolymerization is slower and the purification process is straightforward. The appearance of characteristic peaks in the Fourier transform infrared and carbon-13 nuclear magnetic resonance spectra (1733 cm^{-1} and 183.4 ppm, respectively) of the product confirms the presence of the aldehyde functionality in the modified chitosans. The ^1H NMR spectra also revealed the presence of aldehyde groups. Furthermore, the gradual disappearance of the peaks due to aldehyde protons and a concomitant appearance of a new resonance at ~8.05 ppm with increasing pH indicate the formation of Schiff's base between the aldehyde and the free amine groups. The aldehyde-functionalized chitosan prepared with 6 h of reaction time (chitosan-6h) forms a gel *in situ* without any added external crosslinker and it may potentially be useful as a vehicle for drug delivery.

1. Introduction

Chitosan has increasingly been used in the biomedical and pharmaceutical fields (Ravi Kumar, Muzzarelli, Muzzarelli, Sashiwa, & Domb, 2004) due to its biocompatibility, biodegradability and non-toxicity. Chemical modifications have been used to prepare chitosan derivatives with enhanced biological and physicochemical properties. Recently, aldehyde-functionalized chitosans have received considerable interest. The preparation of chitosan derivatives containing aldehyde groups have been achieved by reaction with periodates. The latter selectively cleaves the 2,3-C–C bond and converts chitosan into a dialdehyde derivative (Matsumura, Yokochi, Winursito, & Toshima, 1997; Vold & Christensen, 2005). Aldehyde-functionalized chitosans have also been prepared by reaction with nitrous acid (HNO_2) (Allan & Peyron, 1995; Hirano, Kondo, & Fujii, 1985; Lin & Lin, 2003; Tommeraas, Varum, Christensen, & Smidsrod, 2001). In this method, chitosan undergoes deaminative cleavage of the 1,4-glycosidic bond, producing 2,5-anhydro-D-mannose as the reducing end, which contains an aldehyde group (Supplementary Data, Scheme S1). Hydrogen peroxide was also found to produce chitosan containing 2,5-anhydro-D-mannose (Tian, Liu, Hu, & Zhao, 2004). The main shortcoming of these reactions is that they all lead to extensive depolymerization of chitosan resulting in low molecular weight or oligochitosan derivatives with very limited use for pharmaceutical and drug delivery applications. In addition, since these reactions are carried out in the solution state, the isolation of products is complex and time-consuming.

The use of nitric acid mixtures either with or without $NaNO_2$ has been extensively studied as an oxidant to convert primary alcohols to aldehydes and/or carboxylic acids and secondary alcohols to ketones (Ogata, 1978). Kumar and Yang (2002) reported the use of a mixture of H_3PO_4/HNO_3–$NaNO_2$ to prepare 6-carboxycellulose from cellulose and the site-selective carboxylation observed in these reactions is thought to occur due to the nitrogen oxide gases generated *in situ*. We hypothesize that nitrogen oxides generated similarly *in situ* will react with chitosan and introduce aldehyde/carboxylic acid groups in its structure rendering modified chitosans the ability to self-crosslink and form a gel (through interaction between the amine and aldehyde groups). The successful design of an *in situ* forming gel without the addition of an external cross-linker will lead to the development of biocompatible matrices for drug delivery applications. With this in mind, we investigated the reaction of chitosan with nitrogen oxides in the solid state, characterized the obtained modified chitosans and assessed their ability to self-crosslink and form a gel.

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2. Materials and methods

2.1. Materials

Chitosan (degree of deacetylation 87% as determined by ^{1}H NMR (Lavertu et al., 2003); viscosity-average molecular weight, [M_v], 43,500) was purchased from Polymar Ciência e Nutrição S/A (Fortaleza, Brazil). Other reagents were analytical grade and used as received.

2.2. Reaction of chitosan with nitrogen oxides

First, a Petri dish (diameter: 9.6 cm, height: 1.3 cm) with 10 g of chitosan powder was placed in a chamber (diameter: 14.5 cm, height: 8 cm, volume: 1321.2 cm^3) containing a mixture of HNO_3 (112 mL) and H_3PO_4 (28 mL). A 14:1 ratio (v/w) of the acid mixture to the chitosan was found to be optimal to produce the modified chitosans reported here. $NaNO_2$ (2 g) was then added to the acid mixture and reddish brown gases formed immediately. Next, the chamber was closed and the chitosan sample was allowed to react with the gases for 2, 4, 6, 12, 24, 36 and 48 h. The reacted samples (hereinafter referred to as chitosan-2h, chitosan-4h, chitosan-6h, chitosan-12h, chitosan-24h, chitosan-36h and chitosan-48h, respectively) were removed, washed with acetone (5× 100 mL), and then dried under vacuum at 50 °C for 24 h.

The dried powder (about 10.5 g) was then taken in distilled water (200 mL), stirred for 24 h and then centrifuged. The supernatant (soluble fraction) was lyophilized and the powder (yield > 80%) so obtained was stored in a plastic jar until use.

2.3. Characterization

2.3.1. FT-IR spectroscopy

The Fourier-transform infrared (FT-IR) spectra (4000–400 cm^{-1}) were obtained as KBr pellets on a Nicolet 210 FT-IR spectrophotometer. The OMNIC software was employed for data analysis. The number of scans was 120 and the resolution was 8 cm^{-1}.

2.3.2. NMR spectroscopy

All products were characterized using an Avance-600 Bruker NMR spectrometer (Billerica, MA) operating at a proton frequency of 600 MHz. ^{1}H and ^{13}C chemical shifts were referenced from the residual HOD or TSP signal accordingly. In order to identify suitable solution conditions for NMR measurements, the temperature and polymer concentration were varied from 15 to 70 °C and from 1 to 10% (w/v), respectively. Generally, the products with shorter reaction times required either lower concentration or higher temperature. Wherever comparisons of spectra were required for analysis, the concentration, temperature and the NMR parameters were kept the same for all the samples. The nature and identity of the reaction products were determined by a battery of 1D and 2D homonuclear and ^{1}H–^{13}C heteronuclear experiments [^{1}H, 1D correlated spectroscopy (COSY), ^{13}C, ^{13}C-distortionless enhancement by polarization transfer (DEPT), heteronuclear multiple quantum coherence (HMQC), and heteronuclear multiple bond correlation (HMBC)]. An inverse detection probe (Broad-band Inverse) and gradient-assisted versions of the pulse sequences were used for these 2D experiments. Direct detection ^{13}C measurements were performed with a broad-band observe probe (PABBO). Typical parameters for the NMR experiments were as follows: ^{1}H (TD, 64k; NS, 4k), ^{13}C (TD, 128k; NS, 10,000), ^{13}C-DEPT (TD, 128k; NS, 5000), 1D COSY (TD, 64k; NS, 512), NOESY (TD, 2k; TD1, 256; NS, 32; DS, 32; mixing times, 1.0, 1.5, and 2.0 s), ^{13}C–^{1}H HMQC (TD, 2k; TD1, 128; NS, 32; DS, 128) and ^{13}C–^{1}H HMBC (TD, 2k; TD1, 128; NS, 32; DS, 128). TD, NS, and DS refer to time domain data points, number of scans, and dummy scans, respectively. All NMR data were processed with TOPSPIN 1.3 suite of software programs. 1D ^{1}H data were processed with zero-filling to 64k data points and 0.2 Hz exponential line broadening, whereas ^{13}C spectra were processed with zero-filling to 128k data points and 1.0 Hz of exponential line broadening. The 2D NMR data were processed with the zero-filling to 2048 points and 1024 points in acquisition and second dimension, respectively. Relative numbers of proton signals multiplied by the integral areas were used for the quantification.

2.3.3. Viscosity measurements

The viscosity-average molecular weight, [M_v], of the parent chitosan and modified chitosans was calculated using the Mark–Houwink equation:

$$[\eta] = \kappa[M_v]^{\alpha}$$

where [η] is the intrinsic viscosity, [M_v] is the viscosity-average molecular weight, and κ and α are constants (0.076 and 0.76, respectively (Rinaudo, Milas, & Le Dung, 1993)). Each sample was dissolved in 0.2 M CH_3COONa/0.3 M CH_3COOH to obtain a final concentration of 0.2–0.5 g/dL. The solutions were filtered through a 0.45 μm Millipore filter and 10 mL of the filtrate was pipetted into a Cannon–Fenske viscometer and transferred to a water bath at 25 °C. Efflux times of pure solvent (t_0) and polymer solutions (t) were measured and used to calculate the relative viscosity ($\eta_r = t/t_0$), then the reduced viscosity, $\eta_{red} = (\eta_r - 1)/C$, where C is the concentration of the polymer solution. The reduced viscosity was plotted against the concentration and the intercept, which corresponds to the intrinsic viscosity, $[\eta] = \lim_{c\to 0}(\eta_{red})$, was used to calculate [M_v].

2.3.4. Potentiometric titration

The number of moles (n) of protonated amine groups in each sample was determined by potentiometric titration. Briefly, an accurately weighed amount (0.2 g) of each modified chitosan sample was dissolved in 25 mL of standardized 0.05 N HCl and the resulting solution was titrated with a 0.1 N NaOH (standardized) solution using a pH-stat titration system. The pH was measured following each addition of the titrant. The same procedure was repeated with the deprotonated modified chitosan samples, which were prepared by treatment with sodium hydroxide (pH 10), followed by washing with distilled water until the pH was 6–7. The pH values thus obtained were plotted against the corresponding volumes of NaOH and a typical curve with two inflection points was obtained, where the first and second points represent the titration of the excess of acid in solution and the chitosan's amine groups, respectively. The number of moles of protonated amine groups in each sample (mol/g) was calculated from the first inflection point determined for the samples treated with (deprotonated) and without (protonated) NaOH according to the following equation:

$$n = \frac{C_1(V_2 - V_1)/1000}{W}$$

where C_1 is the concentration (N) of the NaOH solution, W is the weight of each sample (g) and V_1 and V_2 are the volumes (mL) of the first inflection points of the protonated modified chitosans and their respective deprotonated form, respectively.

2.4. Determination of water-solubility

The aqueous solubility of the modified chitosans was determined by incrementally adding small amounts of the samples to 2 mL of distilled water until no more dissolution occurred. The suspension was stirred for 48 h at room temperature and any remaining undissolved solid was eliminated by centrifugation. A 0.5 mL aliquot of the saturated solution was pipetted into a Petri dish and dried under vacuum at 50 °C for 24 h. The resultant

membrane was weighed and the solubility was calculated by the following equation:

$$\text{Solubility (mg/mL)} = \frac{\text{Weight of the dry membrane}}{0.5}$$

2.5. Preparation of the gels

The self-crosslinked gels were prepared simply by dissolving appropriate amounts of the freeze-dried chitosan-6h in distilled water at room temperature.

3. Results and discussion

3.1. Reaction of chitosan with nitrogen oxides

Since the HNO_3/H_3PO_4–$NaNO_2$ mixture and chitosan are isolated within the reaction chamber, the nitrogen oxide gases diffuse and react with chitosan in dry solid state. As a result, the reaction is slow and readily controllable (*vide supra*) on contrary to the existing methods of oxidation (Allan & Peyron, 1995; Lin & Lin, 2003; Tommeraas et al., 2001).

3.2. Nuclear magnetic resonance analysis

The ^{13}C NMR spectra of chitosan-6h and chitosan-24h show all the signals typical of parent chitosan, i.e. C2 (58.7 ppm), C6 (62.8 ppm), C3 (73.1 ppm), C5 (77.6 ppm), C4 (79.3 ppm) and C1 (100.6 ppm) (Fig. 1). However, at longer reaction times (36 and 48 h), the disappearance of some of these peaks was evident (spectra not shown), which may primarily be due to extensive degradation of chitosan backbone. The resonance at 177.8 ppm was incorrectly assigned to the C6-carboxylic acid group in earlier studies (Qin, Du, Xiao, Liu, & Yu, 2002; Terada et al., 2003), but it actually corresponds to the carbonyl carbon of the N-acetyl group (–NHC̲OCH$_3$) in the partially deacetylated chitosan. Note that the resonance at 177.8 ppm is also present in the spectrum of the parent chitosan (Fig. 2A). The validity of this assignment was also confirmed by HMQC and HMBC experiments (Fig. 2B and C).

Although the ^{13}C spectra of the parent and the modified chitosans (chitosan-6h and chitosan-24h) were similar, some marked differences were observed. The most evident new carbon resonance appeared at ~183.4 ppm in the spectra of modified chitosans, which

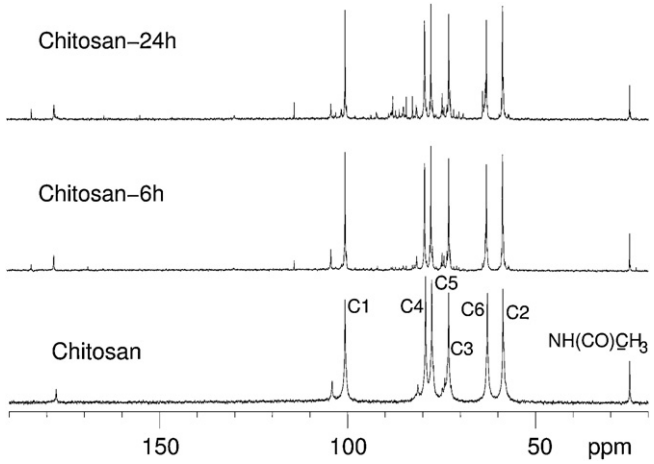

Fig. 1. ^{13}C NMR spectra of parent chitosan, chitosan-6h and chitosan-24h. The NMR samples were prepared at a concentration of 4% (w/v) by dissolving appropriate amounts of the solid in a solution of DCl/D_2O 4% (v/v). The resonance assignments are marked.

was absent for the parent chitosan. This resonance is attributed to the aldehyde carbon (–C̲HO) (Tommeraas et al., 2001) in accordance with the HMQC spectra (Fig. 2D). Fig. 2A also shows an additional signal at 177.7 ppm in the case of chitosan-24h. Although absent in the chitosan-6h sample, it is plausible that this signal could be from the carboxylic acid group originated from the oxidation of the –C(6)H_2OH. Similar result was also found by others (Bordenave, Grelier, & Coma, 2008; Fan, Saito, & Isogai, 2009; Kato, Kaminaga, Matsuo, & Isogai, 2004; Muzzarelli, Muzzarelli, Cosani, & Terbojevich, 1999) after TEMPO-mediated oxidation of chitosan.

To further verify the presence of aldehyde groups in the modified chitosans and its ability to form Schiff's base with the amine groups at C-2 position, the ^1H NMR spectra of chitosan-6h and -24h were obtained at pH 2.0, 4.0 and 6.0 (Fig. 3). The resonances due to the aldehyde proton (–CH̲O) appearing at ~9.36 ppm showed significantly reduced intensity at pH 6. In addition, the observation of a new signal (8.05 ppm; –CH̲=N–, imine) at pH 6.0 supports the formation of Schiff's base between the aldehyde and amine groups at higher pH (Tommeraas et al., 2001). Since the pK_a of chitosan is ~6.5, almost all the amine groups existed in the protonated form at pHs 2.0 and 4.0. However, the fraction of the free amine groups

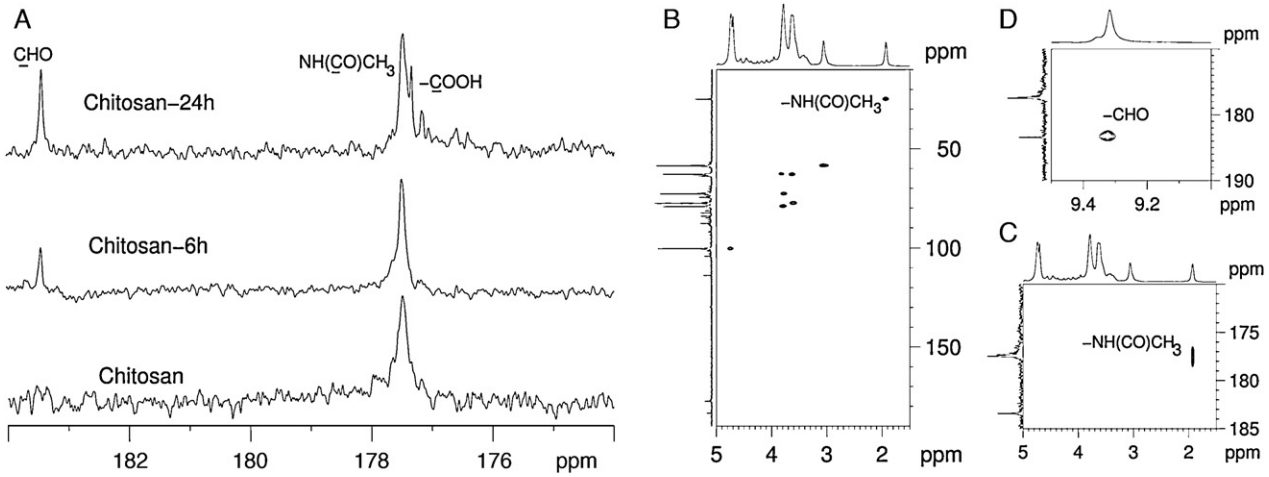

Fig. 2. Expanded ^{13}C NMR spectrum (174–184 ppm) of parent chitosan, chitosan-6h and chitosan-24h (A); ^1H–^{13}C HMQC (B and D) and HMBC (C) spectral cross-sections of chitosan-24 h. The arrows in Fig. 2B–D represent the correlations between the underlined protons and carbons.

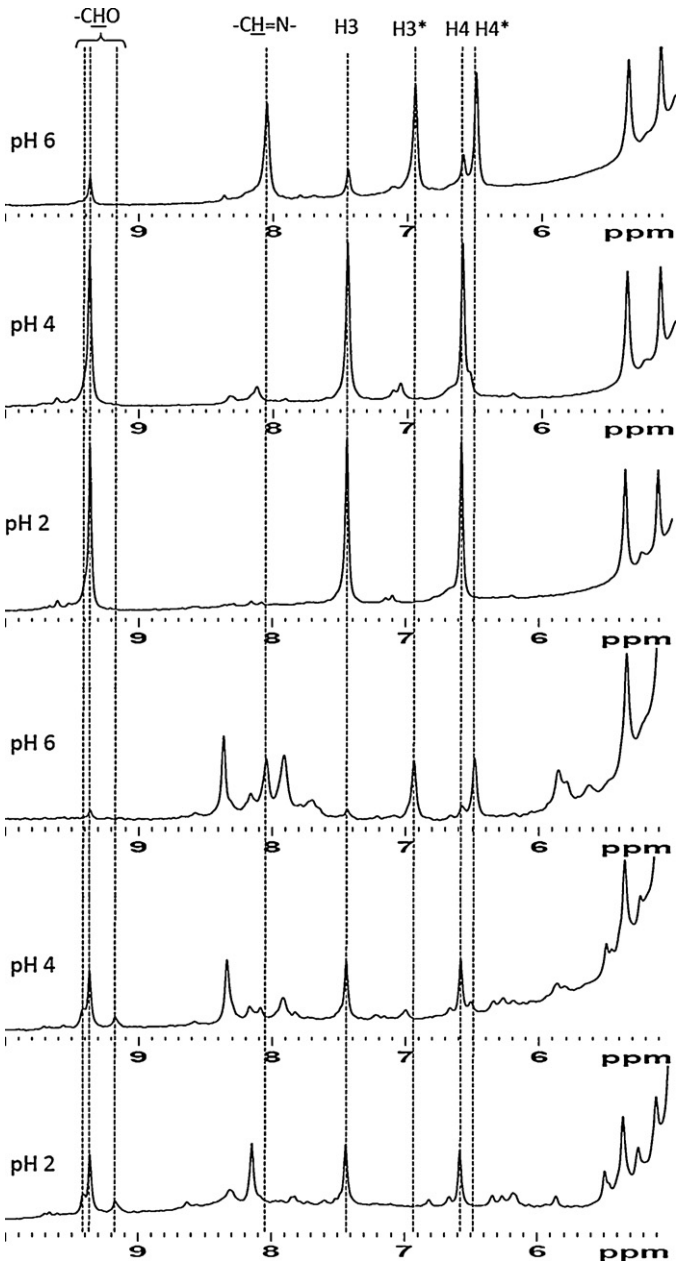

Fig. 3. ^{1}H NMR spectra of chitosan-24h (top 3 traces) and chitosan-6h (bottom 3 traces) in D_2O at pHs 2.0, 4.0, and 6.0.

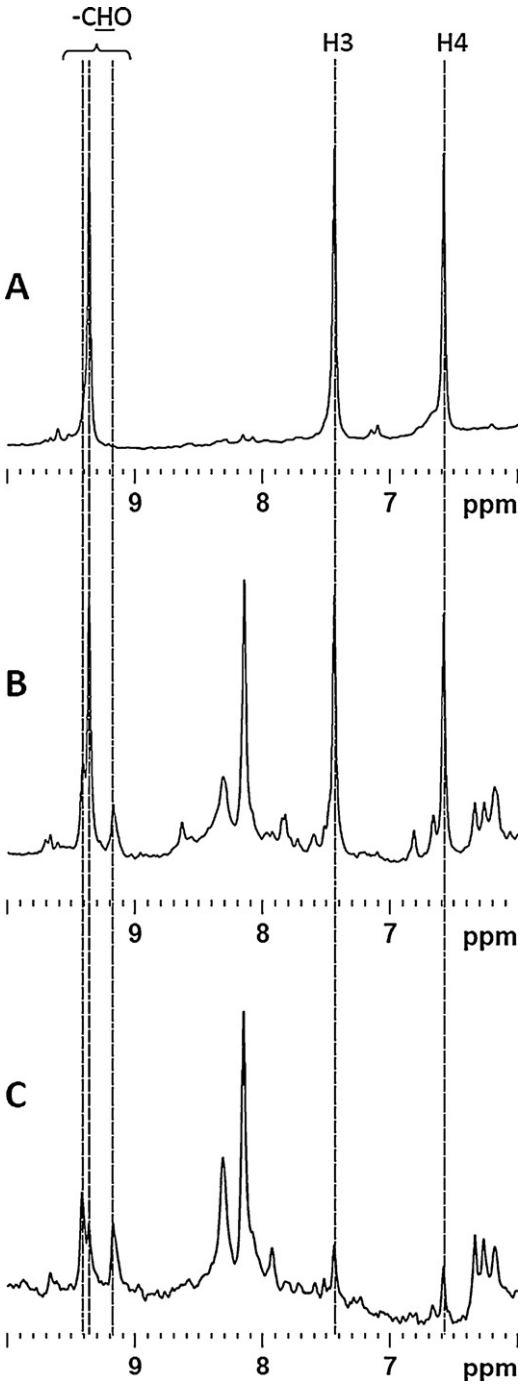

Fig. 4. ^{1}H NMR spectra of chitosan-24h (A), chitosan-6h (B) and chitosan-6h after dialysis in distilled water (C). The pH for all the samples was 2.0.

increased significantly at pH 6.0 favoring the formation of Schiff's base with the aldehydes. These results are in good agreement with those reported by Tommeraas et al. (2001).

Fig. 3 also shows resonances at 7.45 and 6.59 ppm at pH 2.0 and 4.0, which can be attributed to the H3 and H4 protons of 5-hydroxymethyl-2-furfural (HMF) respectively (Supplementary Data, Scheme S1) (Lin & Lin, 2003; Tommeraas et al., 2001). In order to confirm the presence of HMF in the modified chitosan samples, two-dimensional homonuclear and heteronuclear data were collected (Supplementary Data). The J-correlations observed between the resonances at 7.45 and 6.59 ppm in COSY and ^{1}H–^{13}C correlations in HMQC and HMBC data were consistent with the presence of HMF. The intensity of the H3 and H4 resonances also decreased as the pH increased with the appearance of new resonances at 6.48 and 6.94 ppm at pH 6.0. These new resonances are consistent with the formation of Schiff's base complex between HMF and modified chitosan.

Furthermore, the proton spectrum of the chitosan-6h at pH 2.0 (Fig. 3) shows three resonances attributable to aldehyde protons (9.17, 9.36 and 9.41 ppm), whereas the spectrum of chitosan-24h reveals only a single resonance (9.36 ppm). The assignment of these resonances to aldehyde protons is consistent with a typical ^{1}H-chemical shift region of aldehydes, DEPT spectrum accounting for a CH group and a correlation to a carbonyl carbon in the HMQC spectrum. We hypothesize that the chemically and magnetically nonequivalent aldehyde proton resonances observed for chitosan-6h arise from the 2,5-anhydro-D-mannose unit, HMF and possibly from –C(6)HO due to the oxidation of $–C(6)H_2OH$.

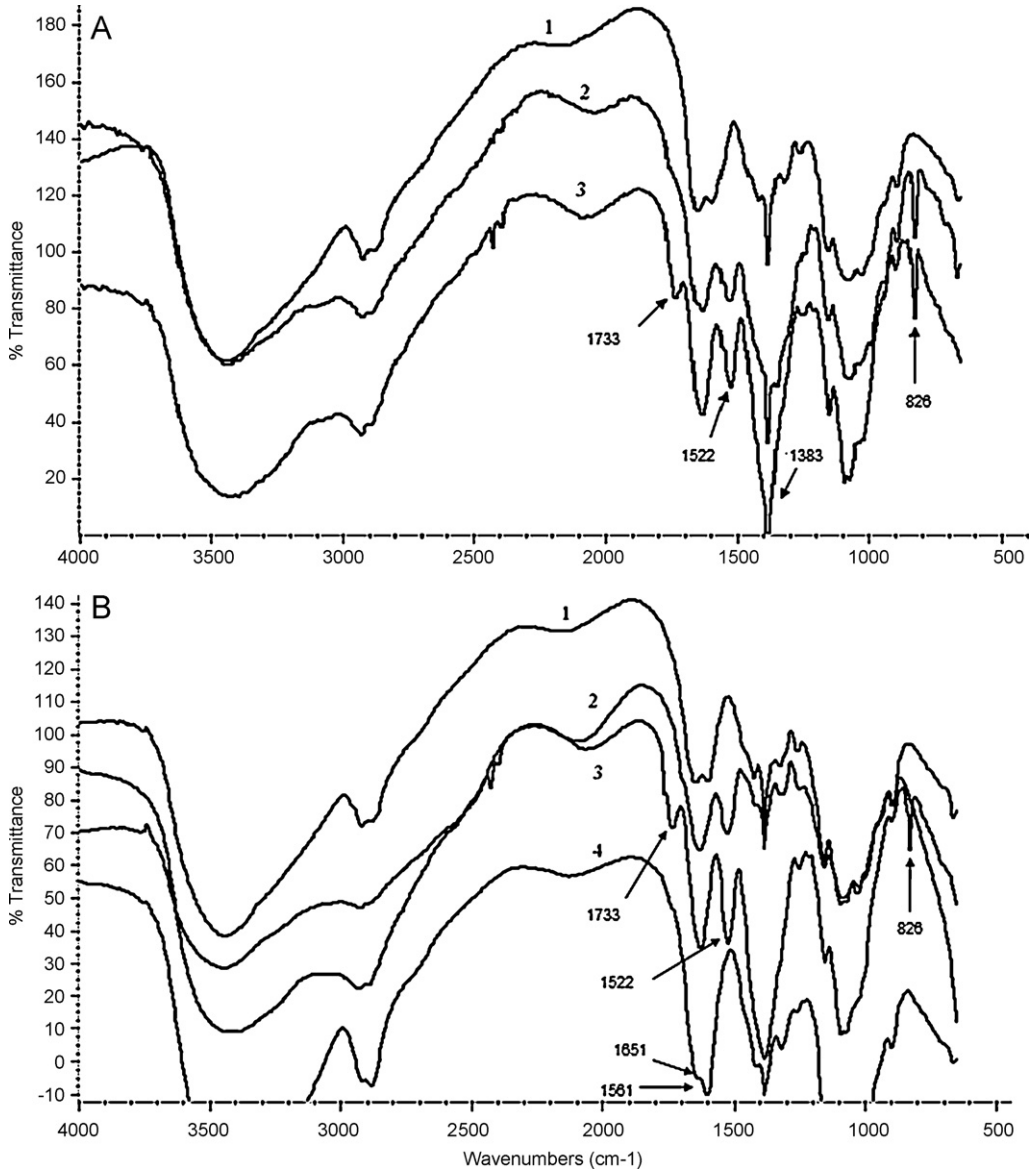

Fig. 5. Fourier-transform infrared spectra of (A): chitosan (1), chitosan-6h (2) and chitosan-24h (3); and (B): chitosan (1), chitosan hydrochloride (2), chitosan-24h (3) and chitosan-24h treated with sodium hydroxide (4).

In order to further determine the nature of the aldehyde groups present in the modified chitosans, the chitosan-6h sample was dissolved in water and the pH was adjusted to 2.0. The solution was then dialyzed against 5× 500 mL of water for 24 h, with the rationale of eliminating the free HMF. Note that the free amine groups are protonated at pH 2.0 and does not form Schiff's base. The dialyzed sample was freeze-dried and the ^{1}H NMR spectrum was obtained in D_2O (Fig. 4C). The intensities of the H3 and H4 resonances of HMF and that of –CHO at 9.36 ppm in the dialyzed sample are significantly lower than those of the non-dialyzed sample (Fig. 4B) indicating that most of the HMF was eliminated after dialysis. On the other hand, the resonances (9.17 and 9.41 ppm) of the other aldehydes are still present in the spectrum of the dialyzed sample and appear similar in intensities. In addition, the aldehyde resonance (9.36 ppm) that decreased in intensity after dialysis is exactly the same as the one that is also present in the chitosan-24h spectrum (Fig. 4A) indicating that at longer reaction times (24h and longer) the conversion of 2,5-anhydro-D-mannose to HMF is facilitated.

3.3. Fourier-transform infrared spectroscopic analysis

Fig. 5A shows the FT-IR spectra of the parent chitosan, chitosan-6h and chitosan-24h. The chitosan spectrum reveals a band at 3423 cm^{-1} characteristic of the –OH group stretching vibration, two bands at 1651 cm^{-1} and 1559 cm^{-1} for the stretching and bending vibrations of amide I and $–NH_2$ respectively and a band at 1384 cm^{-1} corresponding to the stretching vibration of the $–CH_3$ groups (Supplementary Data). On the other hand, the spectra of chitosan-6h and chitosan-24h show two additional bands at 1522 and 1733 cm^{-1}, which are characteristic of $–NH_3^+$ and –CHO, respectively. The 1733 cm^{-1} band appears as a shoulder in the chitosan-6h sample, but its intensity is significantly higher for chitosan-24h.

The FT-IR spectrum of NaOH-treated chitosan-24h further confirms the presence of protonated amine in the modified chitosans. Fig. 5B shows the FT-IR spectra of chitosan, chitosan hydrochloride, chitosan-24h as produced and after the treatment with NaOH. The 1522 cm^{-1} band was absent in the NaOH-treated chitosan-24h, but

a new peak appeared at ~1561 cm^{-1}, attributable to the free amine groups. On the other hand, the spectrum of chitosan hydrochloride is similar to that of the chitosan-24h. Since $NO_{2\ (g)}$ is generated *in situ* (Kumar & Yang, 2002) and there was an abundance of water vapor in the reaction chamber, the acid droplets produced from the gas-phase reaction of water vapor and nitrogen dioxide reaction protonated the free amine groups of the modified chitosans (England & Corcoran, 1974).

The FT-IR spectra of the modified chitosans (Fig. 5A) also show an increase in the intensity of the 1384 cm^{-1} band and the appearance of a new band at 826 cm^{-1}. These bands have been assigned to NO_3^- ions and are in good agreement with earlier results reported in literature (Huo et al., 2008; Pakula, Swiatkowski, Walczyk, & Biniak, 2005; Yang, Zhou, Chuo, Wang, & Yu, 2007); Huo et al. (2008) synthesized chitosan nitrate salts by mixing chitosan with a solution of nitric acid and acetone, whereas Yang et al. (2007) oxidized chitosan films by dipping it in glacial acetic acid solution saturated with NO_2 gas. In both studies, the intensities of the peaks in the 1380–1384 cm^{-1} region increased due to the overlap of N=O vibration of NO_3^- with $-CH_3$ symmetric deformation. Moreover, the new peak around 826 cm^{-1} in the spectra of our modified chitosans is also found in both studies, in which the authors also attributed to the presence of NO_3^- in the chitosan molecule. In fact, the intensity of both peaks decreased after treating chitosan-24h with sodium hydroxide (Fig. 5B), confirming that the chitosan-6h and chitosan-24h obtained in the present study exist as nitrate salts.

The treatment of chitosan-24h with NaOH also led to a significant reduction of the 1733 cm^{-1} band (Fig. 5B). This is consistent with the explanation that the aldehyde content in chitosan-24 h arises primarily due to HMF, and extensive washing performed with water to remove excess NaOH prior to collecting the FT-IR spectrum eliminated most of the free HMF. However, the formation of imine complex between HMF and free amine groups of chitosan cannot be completely ruled out as an explanation for the decrease in intensity, considering the NMR results (Fig. 3, resonances due to H3* and H4*).

The presence of absorption bands at 1152 cm^{-1} (asymmetric stretching of C–O–C bridge), 1072 and 896 cm^{-1} (C–O stretching) in the spectra of chitosan-6h and chitosan-24h (Fig. 5A) demonstrates that the backbone structure of modified chitosans remains unaltered from the parent chitosan.

3.4. Quantitation of protonated amine groups

The protonation of the amine groups in the modified chitosans was also confirmed by titration experiments as shown in Fig. 6A. The protonation increased with increasing the time of exposure to nitrogen oxides and it is consistent with the reaction of chitosan with the acid droplets originated from the reaction of the water vapor present inside the sealed chamber and the nitrogen oxides (England & Corcoran, 1974; McKinnon, Mathieson, & Wilson, 1979).

3.5. Viscosity-average molecular weight

The main drawback of the currently available methods to produce aldehyde-containing chitosan is the fast depolymerization; the resulting chitosan oligomers are only marginally useful for drug delivery applications. Since we aim to produce an *in situ* forming gel system through the self-crosslinkage of the amine and aldehyde groups the molecular weight of the modified chitosans should be taken into account. The viscosity-average molecular weights of chitosan and modified chitosans were determined using the Mark–Houwink constants, α and κ, reported by Rinaudo et al. (1993). Since the solution conditions and the chitosan (degree of deacetylation, DD, 87% vs 88.5%) used in our study are very similar

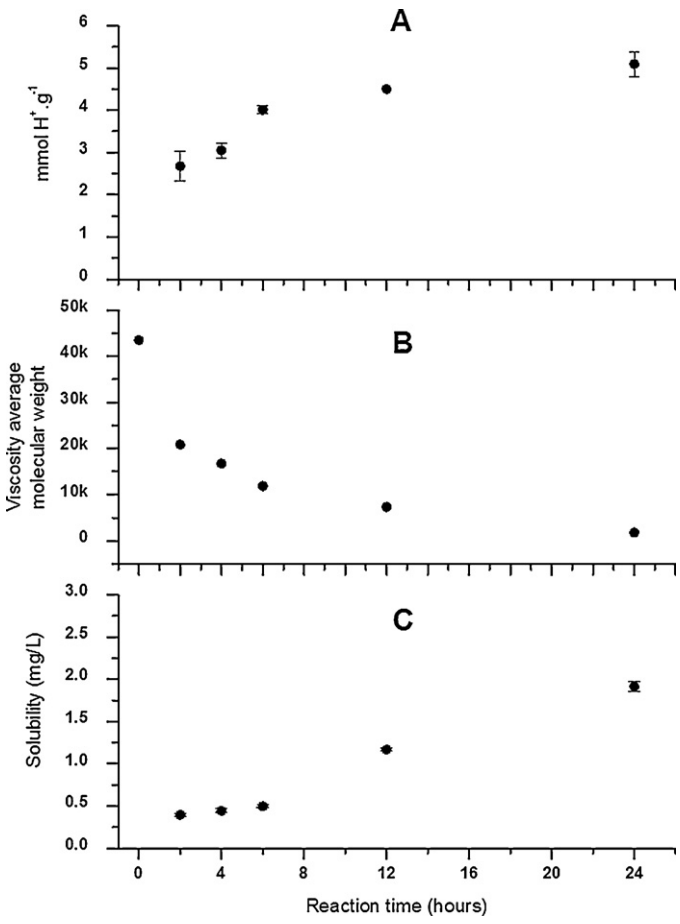

Fig. 6. Effect of the time of exposure (reaction time) to the nitrogen oxides on the protonation of the amine groups (A), viscosity-average molecular weight (B) and water solubility (C) of the parent chitosan. The moles of H^+ were measured by using the titration method and it represents the excess of H^+ on each modified chitosan compared to the respective deprotonated sample. The [M_v] of all the samples were determined using the Mark–Houwink equation. Each point and its respective error bar correspond to an average of 3 samples and the standard deviation, respectively.

to those reported by these authors, we used α and κ values of 0.76 and 0.076 to determine [M_v] of chitosan and modified chitosans. It is noted, however, that the degree of deacetylation of the modified chitosans is expected to be less than that of the parent polymer due to the deamination step involved in the reaction forming the 2,5-anhydro-D-mannose (Tommeraas et al., 2001).

The relationship between [M_v] and reaction time is depicted in Fig. 6B. The [M_v] of the chitosan (shown in the plot at 0 h of reaction) decreased by more than 50% after 2 h of reaction and continued to decrease with time, indicating that the [M_v] of the modified chitosans can be controlled by varying the reaction time. The degree of polymerization (DP) for chitosan-24h is ~11. On the other hand, a decrease in the molecular weight from approximately 100,000 to less than 10,000 occurred after reacting chitosan with H_2O_2 for 30 min (Tian et al., 2004). The use of $NaNO_2$ in the presence of HCl has also been found to quickly depolymerize chitosan. Mao et al. (2004) found a decrease of ~50% in molecular weight (200 kDa, 85% deacetylated) after 1 h of reaction, whereas Tommeraas et al. (2001) reported a maximum DP of 5 for their soluble chitosan oligomers prepared after 12 h of reaction. Lin and Lin (2003) obtained modified chitosans with DP ranging from 4 to 11 after allowing a high molecular weight chitosan (630,000, 82% deacetylated) to react with $NaNO_2$ in the presence of acetic acid for 3 h. In the present study, the chitosan-4h and chitosan-12h samples had a DP of around 110 and 45 respectively, which indicates that

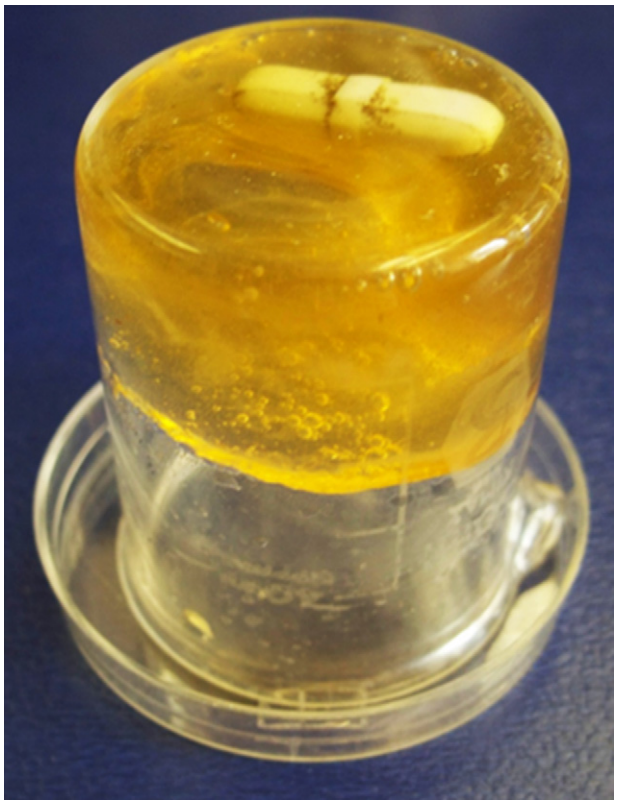

Fig. 7. Self-crosslinked modified chitosan gel produced by dissolving 6% (w/w) of the chitosan-6h in distilled water (pH ~5.5). The beaker was placed upside down showing the gel holding the stirring bar in place revealing its mechanical stability.

the method used in this study is milder and offers a better control on the degree of depolymerization.

3.6. Water-solubility of modified chitosan

Fig. 6C shows that the aqueous solubility of modified chitosans was directly proportional to the reaction time. The increase in the solubility was evident even after 2 h of reaction, compared to the untreated chitosan (shown in the plot at 0 h of reaction time). Since chitosan-6h formed a gel while attempting to dissolve it, the solubility value reported for this sample is only an estimate. The gradual increase in the water solubility of the modified chitosans with reaction time is a result of the decrease in the $[M_v]$ (Fig. 6B) combined with the increase in the amine protonation (Fig. 6A).

3.7. Preparation of the self-crosslinked chitosan gels

Among the various modified chitosans investigated, only the chitosan-6h is found to form a gel. Chitosan-6h has three distinct aldehyde functionalities as revealed by the pH-dependent NMR spectroscopy and their differences in nature and quantity in comparison to the other chitosan analogs are particularly important for the formation of the self-crosslinked gel. Chitosan-2h and chitosan-4h are unable to form gels due to the fact that their molecular weights and degrees of protonation are not suitable for their dissolution in water and the number of aldehydes in these samples is limited to form adequate cross-linkages. On the other hand, chitosan-12h and chitosan-24h samples do not form gels, since they cannot sustain the necessary three-dimensional structure, as a result of substantial depolymerization.

The gels of chitosan-6h were obtained by simply dissolving a minimum of 6% (w/w) of this sample in distilled water under stirring and they were clear and transparent as shown in Fig. 7. The formation of the self-crosslinked gel involves the dissolution of the sample and the formation of Schiff's base leading to gelation. Consequently, the rate of *in situ* crosslinking and gel formation is determined by the underlying chemical kinetics of the crosslinking reaction, the ease of diffusion of the chitosan-6h through the viscous pre-gel solution, and the concentration of the chitosan-6h. On the contrary, the exiting methods of the preparation of chitosan gels involve the addition of an external agent such as glutaraldehyde to provide aldehyde functionalities for crosslinking. The main disadvantage of external agents is its toxicity to human tissues even in trace amounts (Berger, Reist, Mayer, Felt, & Gurny, 2004). Thus, the gels produced in this study do not require the addition of an external crosslinker and it has the potential to be developed into a more biocompatible matrix for drug delivery applications. The stability and the water uptake capacity as well as the rheological and drug release properties of these gels are currently being investigated in our laboratory.

4. Conclusions

The reaction of chitosan in solid state with nitrogen oxides generated from a HNO_3/H_3PO_4–$NaNO_2$ mixture is demonstrated to be an optimal method for the preparation of modified chitosans with aldehyde functionality. This method is milder than the existing methods and the pyranose skeleton of chitosan was preserved up to 24 h of reaction. The ^1H and ^{13}C NMR and FT-IR analysis of chitosan-6h and chitosan-24h revealed unequivocally the presence of aldehyde functionalities. The aldehyde groups are clearly distinguishable from the carboxylic or keto functionalities by the ^{13}C–^1H correlation peaks in the HMQC spectrum and by the ^{13}C-DEPT spectrum; the latter would not show any ^{13}C resonance in the carbonyl spectral region, if there were no aldehyde groups present. Though carbonyl-^{13}C spectral regions of chitosan-6h and chitosan-24h are very similar, the ^1H resonances showed marked differences. First, three ^1H resonances in the case of chitosan-6h identify three chemically and magnetically distinguishable aldehyde groups; in comparison, the observation of a single resonance for chitosan-24h can be attributable only to the presence of one type of aldehyde group. The pH-dependent ^1H spectra of chitosan-6h with and without dialysis further revealed one of the aldehyde ^1H resonances is due to HMF, which is the same as that of chitosan-24h. The substantial reduction of the intensity of this resonance compared to the remaining two resonances of chitosan-6h after dialysis deduces that at reaction times of 24 h and longer, the aldehydes present are primarily due to HMF, as a result of depolymerization followed by the cleavage into 2,5-anhydro-D-mannose and subsequently to HMF at low pH. The FT-IR analysis and potentiometric titration experiments demonstrated that the amines are protonated and the degree of protonation increased with reaction time, whereas the viscosity and solubility measurements revealed gradual depolymerization. Hence, the cleavage of low-molecular weight chitosans to 2,5-anhydro-D-mannose and in turn to HMF is rather a result of the reduction in molecular size than the protonation state. The chitosan-6h is found to have an optimal structural architecture and adequate aldehyde and amine functionalities to self-crosslink and form gels readily in water without the addition of an external crosslinker.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.carbpol.2011.09.090.

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