Carbohydrate Polymers 90 (2012) 894–900

Contents lists available at SciVerse ScienceDirect

Carbohydrate Polymers

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

Rheological, water uptake and controlled release properties of a novel self-gelling aldehyde functionalized chitosan

Eduardo P. Azevedo*, Vijay Kumar

Department of Pharmaceutical Sciences and Experimental Therapeutics, The University of Iowa, Iowa City, IA, 52242, USA

ARTICLE INFO

Article history:
Received 11 May 2012
Received in revised form 8 June 2012
Accepted 9 June 2012
Available online 17 June 2012

Keywords:
Aldehyde-functionalized chitosan
Self-crosslinkable gel
Rheological properties
Water uptake
Drug release

ABSTRACT

In this work, aldehyde-functionalized chitosan was used to prepare gels through Schiff base formation between the aldehyde and the free amine groups. The gels, which were formed *in situ* without an external crosslinker, were characterized in respect to their rheological properties and water uptake capacity. In addition, the potential use of these gels as drug carriers was investigated using metronidazole (Mw 171 Da), FTIC-dextran (Mw 40 kDa) and bovine serum albumin (Mw 66 kDa), as the model drugs. Higher concentrations of the aldehyde-functionalized chitosan originated more rigid gels, as evidenced by the higher values of complex modulus (G^*). The highly porous structure of these gels allowed for more than 400% of water uptake. The release of the model drugs followed a near zero-order release profile. The molecular size as well as the pH of the medium influenced the amount of drug released, where the higher the pH, the slower the resulting drug release.

1. Introduction

Chitosan, the partially acetylated (1–4)-2-amino-2-deoxy-β-D-glucan (Muzzarelli et al., 2012), is easily obtained by the deacetylation of chitin, the second most abundant polysaccharide found in nature as a component of exoskeletons of crustaceans and insects (Prabaharan, 2008; Ravi Kumar, 2000; Rinaudo, 2006). Due to its biocompatibility, biodegradability and non-toxicity, chitosan has increasingly been used in the pharmaceutical field (Ravi Kumar et al., 2005). Because of the presence of free amine groups in its structure, chitosan has good crosslinking ability and swelling properties, which makes this polymer an optimum material for preparing gels (Singh, 2006). Chitosan gels are usually prepared through interchain interaction of the free amine groups, which is achieved by using a crosslinker agent (*e.g.* glutaraldehyde) (Berger, Reist, Mayer, Felt, & Gurny, 2004). The crosslinkage occurs through Schiff base reaction between the aldehyde and the free amine groups of glutaraldehyde and chitosan, respectively. However, evidence of the toxicity of glutaraldehyde to humans has limited its use to prepare chitosan gels for biomedical applications (Hoare & Kohane, 2008; Muzzarelli, 2009).

Chitosans carrying aldehyde functions have been prepared by our group (Azevedo, Santhana Mariappan, & Kumar, 2012) following the method previously reported by (Kumar & Yang, 2002) to oxidize cellulose. In our study, nitrogen oxide gases generated similarly *in situ* reacted with chitosan in the solid state producing water soluble aldehyde-functionalized chitosans in high yield, where the depolymerization was slower than other methods previously reported. We have shown that the introduction of aldehyde groups in chitosan's structure rendered modified chitosans the ability to self-crosslink and form a semi-solid gel (through Schiff base formation). The production of chitosan gels without the addition of crosslinking agents would enable the development of biocompatible chitosan matrices for drug delivery application. Therefore, the aim of the present work is to optimize the production of self-crosslinked chitosan gels and determine its rheological properties and water uptake capacity. Furthermore, the potential application of these gels as drug carrier was evaluated using model drugs with different molecular weights.

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], 44 kDa) was purchased from Polymar Ciência e Nutrição S/A (Fortaleza, Brazil). Other reagents were analytical grade and used as received.

2.2. Preparation of the aldehyde-functionalized chitosan

Aldehyde-functionalized chitosan was prepared according to the method described in a paper recently published by our group

* Corresponding author. Tel.: +1 319 335 1336; fax: +1 319 335 1270.
E-mail address: eduardo-azevedo@uiowa.edu (E.P. Azevedo).

0144-8617/$ – see front matter © 2012 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.carbpol.2012.06.017

(Azevedo et al., 2012). Briefly, 10 g of chitosan (powder) was finely spread in a Petri dish (diameter: 9.6 cm, height: 1.3 cm) and 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). The uniform distribution of that amount of chitosan over the surface of the petri dish with the aforementioned diameter provided a very thin layer of powder. $NaNO_2$ (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 6 h. The obtained aldehyde-functionalized chitosan, hereinafter referred to as chitosan-6h, was 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 so obtained was stored in a plastic jar until use.

2.3. Preparation of chitosan-6h gel

The self-crosslinked gels were prepared by dissolving appropriate amounts of the freeze-dried chitosan-6h, equivalent to give final concentrations of 6, 6.5 and 7% (w/w), in distilled water and allowed to stir at room temperature.

2.4. Rheological properties

In this study, the oscillatory viscoelastic measurements were performed using a rheometer (Haake RheoStress 1 Rotational Rheometer, Haake Mess-Technik GmbH. Co., Karlsruhe, Germany), equipped with a 35 mm parallel-plate and a RheoWin Pro Data Manager (version 2.96) data acquisition software. The experiments were performed at 37 °C, where ~1 g of the chitosan-6h gel (6, 6.5 and 7%, w/w) as well as chitosan-6h 5% (w/w) solution was carefully placed over the bottom plate. Then, the top plate was brought down until a distance of 1.0 mm between the plates was reached. A solvent trap was used to cover the sample and minimize the loss of water.

The viscoelastic parameters determined by oscillatory measurements were the storage or elastic modulus (G'), loss or viscous modulus (G''), complex modulus (G^*) and the loss tangent ($\tan\delta$). The complex modulus (G^*) represents the overall contribution of each modulus and is mathematically defined as $G^* = (G'^2 + G''^2)^{1/2}$, whereas the loss tangent is $\tan\delta = G''/G'$ (Deem, 1988; Schott, 2000). The viscoelastic parameters were determined as a function of frequency (f) as a result of a frequency sweep. However, in order to ensure that the obtained viscoelastic parameters are a function of only frequency, an oscillation stress sweep was also performed, where G' and G'' were measured as a function of applied shear stress and the linear viscoelastic region (defined as a variation of less than 5% in the G' and G'' according to the applied stress) was determined. In this study, an oscillation stress sweep (1–200 Pa at a fixed frequency of 1 Hz) was performed on the gels and the linear viscoelastic region identified. Thus, a stress value of 60 Pa was selected for the frequency sweep tests as it was the midpoint of the linear region. Frequency sweep tests were conducted from 0.08 to 1.6 Hz.

2.5. Scanning electron microscopy (SEM)

The morphology and porous structure of the chitosan-6h gels were obtained by SEM. Samples were frozen in dry ice at −80 °C and lyophilized for 48 h. Pieces were cut from the lyophilized samples, mounted on stubs and sputter coated using K550 Emitech Sputter Coater (Emitech Ltd., Kent, England). Each sample was imaged at 3.0 kV on a Hitachi S-4800 field emission scanning electron microscope (Hitachi High-Technologies Corporation, Tokyo, Japan).

2.6. Determination of water uptake capacity (WUC)

To determine the pH-dependent water uptake capacity of the freeze-dried gels, an accurately weighed amount of each sample was immersed in citrate buffer (pH 4.2) and phosphate buffer (pH 7.4) at 37 °C. At pre-determined time intervals, swollen samples were removed and the excess of water was blotted from the surface with a filter paper and then weighed. The water uptake capacity (WUC) was determined (in percentage) according to the following equation:

$$\mathrm{WUC}(\%) = \frac{\text{weight of the wet sample} - \text{weight of the dry sample}}{\text{weight of the dry sample}} \times 100$$

2.7. Drug release studies

In order to access the potential use of chitosan-6 h gels as drug carrier, metronidazole (Mw 171 Da), FITC-dextran (Mw 40 kDa) and bovine serum albumin (BSA) (66 kDa) were used as model drugs. Gels containing each of these drugs were prepared by dissolving an appropriate amount of chitosan-6h in a previously prepared aqueous solution of the drug, yielding final concentrations of 6 and 7% (w/w) of the chitosan-6h. Gels containing metronidazole or FITC-dextran were prepared at a concentration of 0.4% (w/w) in respect to the total weight of the gel. However, due to its low limit of detection, BSA containing gels were prepared at a solute concentration of 4% (w/w).

An accurately weighed amount of each gel was then placed in the sample holder, which had a circumferential opening in the center equal to 7 cm in diameter. The assembly was immersed in 100 mL of different buffer solutions (citrate, acetate and phosphate, pH 4.2, 5.5 and 7.4, respectively), thermostated at 37 °C and stirred at 50 rpm. At predetermined time points, 2 mL of the release medium were withdrawn (the same volume was replaced with fresh buffer solution) and appropriately diluted. The cumulative release of metronidazole and BSA were determined at 320 and 278 nm, respectively, using a HP 8453 UV–visible spectrophotometer (Hewlett-Packard Company, Waldbronm, Germany) equipped with a Hewlett-Packard HP ChemStation software. On the other hand, the release of FTIC-dextran was determined fluorimetrically (Perkin-Elmer 3000 Fluorescence Spectrometer, excitation 485 nm and emission 520). The percentage of drug released was calculated using standard calibration curves

2.8. Statistical analysis

Significant differences between groups were determined using Student's t-test and one-way analysis of variance (ANOVA) on a computer equipped with Minitab 15 software package (Minitab® Solutions, State College, PA) at a significance level of 5%.

3. Results and discussion

3.1. Preparation of the chitosan-6h gels

The integrity of the chitosan's backbone in the chitosan-6h has been confirmed using ^{13}C NMR and FT-IR spectroscopic methods, as shown in our previous work (Azevedo et al., 2012). Although the [M_v] of the parent chitosan (calculated using the Mark–Houwink equation) dropped from 44 to 12 kDa after 6 h of reaction (chitosan-6h) with the nitrogen oxides, this method is still much milder and

easier to control than the existing methods for preparing aldehyde-functionalized chitosan.

In this study, semi-solid gels containing 6, 6.5 and 7% (w/w) of chitosan-6h (pH values of 5.53, 5.60 and 5.63, respectively), were obtained by simply dissolving appropriate amounts of the freeze-dried chitosan-6h in distilled water under stirring. Since the chitosan-6h is water soluble, no addition of acid was necessary to dissolve the samples.

However, the use of chitosan-6h at a concentration of 5% (w/w) was not enough to form a gel. A viscous solution was obtained at this concentration, which led us to assume that a minimum of 6% was necessary to form the gel. On the other hand, concentrations above 7% could not be used in this study because the gel was formed before the total amount of chitosan-6h was completely dissolved. The formation of the self-crosslinked chitosan gel involves the dissolution of the sample and the formation of Schiff base between the aldehyde and the amine groups in the aldehyde-functionalized chitosan, which leads to gelation. Both process occurred simultaneously. Apparently, as chitosan-6h dissolved in water, the solution became increasingly more viscous until the gel was obtained and no further stirring was possible due to the non mobility of the stirring bar. The obtained gels were clear and transparent, even at higher concentration (7%). However, at concentrations above 7%, fractions of hydrated chitosan-6h were still suspended on the gel and even the determination of the rheological properties of such a gel would be problematic. Therefore, the rate of *in situ* crosslinking and gel formation seems to be 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.

3.2. Rheological properties of the gels

Generally, gels are viscoelastic materials that exhibit viscous flow combined with elastic deformation when stressed. Testing viscoelastic materials at large deformations for relatively long time can break the underlying structures responsible for their rheological properties. In order to avoid this, viscoelastic materials are tested at small, periodic deformations for a short period of time. In a typical viscoelastic measurement, the material is placed in between a cone and plate or two plates and the top plate or cone oscillates at a small fixed amplitude (Deem, 1988; Schott, 2000). On the other hand, dynamic light scattering has recently been used as another non-destructive method to determine the gelification of chitosan solutions by glutaraldehyde (de Morais, Pereira, & Fonseca, 2012). In the present work, oscillatory rheometry, operated in stress and frequency modes was used to characterize the viscoelastic properties of chitosan-6h self-crosslinked gels.

Prior to running the oscillatory frequency sweep test, an oscillation stress sweep was performed on the gels in order to find the linear viscoelastic region. Fig. 1A shows the change in elastic modulus (G') according to the applied shear stress on the chitosan-6h gels (concentrations of 6, 6.5 and 7%). The G' increased with increasing shear stress until a maximum value was reached. Then, as the shear stress kept increasing, G' decreased until it reached almost zero, which means that the three-dimensional network of the chitosan-6h gels was broken. This yield value is called yield stress and when it is exceeded, the gels flow like liquids. Therefore, at stress values bellow the yield stress they produce mainly elastic deformations on gels, whereas at stress values above the yield stress, the network is partially ruptured and flow occurs (Schott, 2000). On the other hand, the initial increase in G' with the shear stress showed in Fig. 1A might be due to an increase in chain entanglement. In addition, the shear stress might have increased the mobility of the chains, which possibly allowed for more interactions between the amine and aldehyde groups. Therefore, the increase in Schiff base

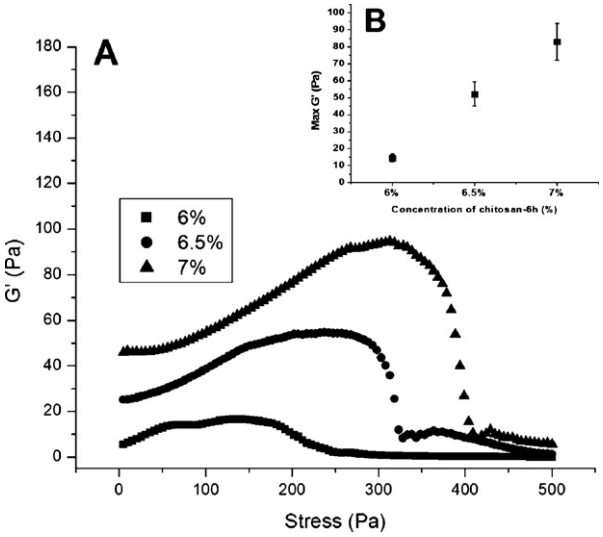

Fig. 1. (A) Elastic modulus (G') *versus* shear stress for chitosan-6h gels at different concentrations. (B) Maximum G' value obtained in the stress range of 0–500 Pa ($n = 3$, error bar = standard deviation).

formation with the increase in shear stress could also be responsible for the increase in G'. It is important to note that the maximum G' reached, which is related to the elasticity and solid-like behavior of the gel, varied according to the concentration of the chitosan-6h in the gel. When the maximum G' of each sample was plotted against the concentration of the chitosan-6h an almost linear relationship ($R^2 = 0.9966$) was observed, as depicted in Fig. 1B.

The crossover between the elastic and viscous moduli (G' and G'', respectively) represent the offset point of the gel formation, where the solid-like characteristic (G') of the gel gets higher than its liquid-like characteristic (G'') (Sahiner, Singh, Kee, John, & McPherson, 2006; Tang, Du, Hu, Shi, & Kennedy, 2007). In our oscillatory stress sweep experiment all samples showed higher values of G' in comparison with G'' at most of the shear stress values. However, G' started to decrease after the yield stress was exceeded, where it eventually gets lower than G'', as shown in Fig. 2A with the chitosan-6h 6.5% (w/w) gel as an example. The point where $G' = G''$ represents the transition from the solid-like behavior to a more liquid-like, which corroborates that the underlying structure of the chitosan-6h gels is broken at high shear stresses. When the stress value at which $G' = G''$ was plotted against the concentration of

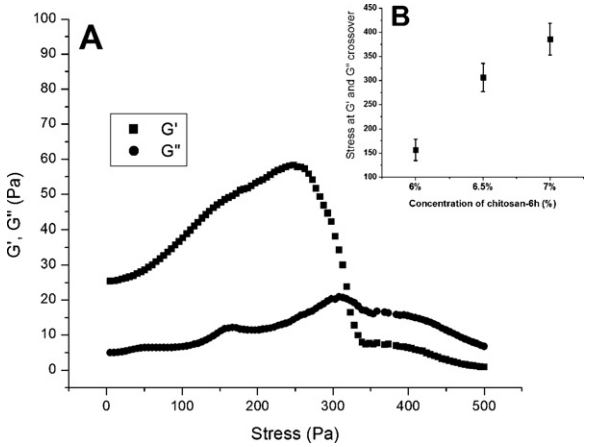

Fig. 2. (A) G' and G'' of chitosan-6h 6.5% gel according to applied shear stress. (B) Plot of the stress value at which G' and G'' cross each other *versus* concentration of the chitosan-6h in the gel ($n = 3$, error bar: standard deviation).

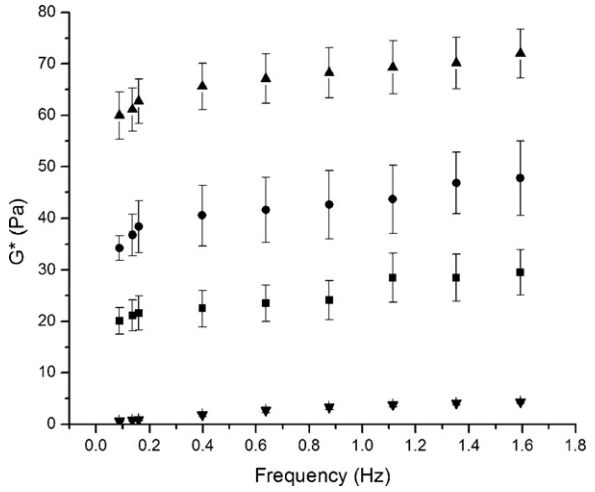

Fig. 3. Frequency sweep from 0.05 to 1.6 Hz for chitosan-6h gels showing the effect of concentration on their complex moduli (G^*) ($n = 3$, error bar: standard deviation).

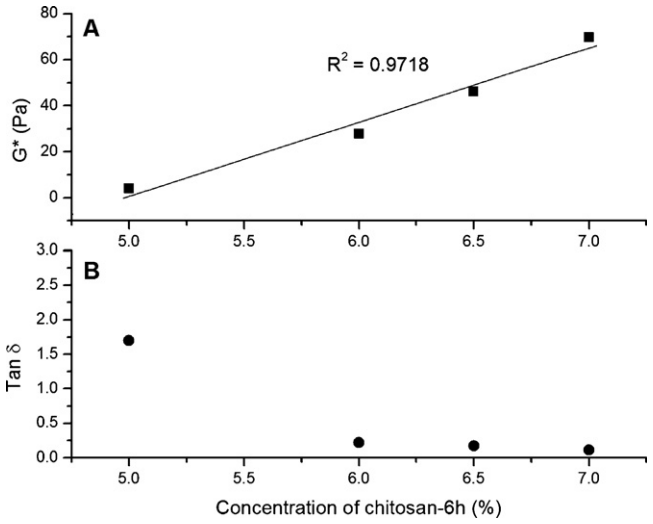

Fig. 4. Complex modulus (G^*) (A) and tan δ (G''/G') (B) as a function of chitosan-6h concentration (5, 6, 6.5 and 7%) measured at a frequency of 1.35 Hz.

chitosan-6h (Fig. 2B) it showed that the stress at which the gel structure is disrupted depends upon the concentration of the chitosan-6h ($R^2 = 0.97$), where the higher the concentration the more elastic the gel becomes.

Fig. 3 shows the change in the complex modulus (G^*) as a function of the frequency applied to the chitosan-6h gels (6, 6.5 and 7%), as well as to the chitosan-6h 5% solution. The change in the frequency had a little effect on the G^*, where it slightly increased with increasing frequency. Since G^* represents the overall rigidity of the sample, it is likely that the strength of the chitosan-6h gel increases with the increase in concentration ($p < 0.05$). Moreover, similar to the stress sweep test results, G' was always higher than G'' for chitosan-6h at 6, 6.5 and 7% for the entire frequency range (data not shown). In addition, the difference between G' and G^* was minimum, which means that G^* is mainly represented by the elastic modulus. On the other hand, the complex modulus (G^*) for the 5% solution was much lower than those for the gels (chitosan-6h 6, 6.5 and 7%). In this case, the viscous modulus was higher than the elastic modulus for the entire frequency range (Supplementary Data), which is characteristic of a viscous solution.

When the G^* value of each sample (measured at 1.35 Hz) was plotted against the concentration of the chitosan-6h, an almost linear relationship ($R^2 = 0.9719$) was obtained (Fig. 4A). On the other hand, tan δ (G''/G') represents the relative viscous to elastic behavior of the sample, where tan $\delta < 1$ indicates the predominance of the elastic component over the viscous one. All chitosan-6h gels at concentrations above 6% showed values of tan δ below 0.25. According to Fig. 4B, tan δ decreased as the concentration of chitosan-6h went from 6 to 7%, which corroborates that the gels become more elastic and solid-like with increasing the amount of chitosan-6h in the gels. However, the chitosan-6h sample at 5% showed a tan δ value of 1.7, which characterizes a predominance of the viscous component.

3.3. Water uptake capacity (WUC)

Gels are three dimensional hydrophilic polymeric networks that absorb and retain water or biological fluids in their structure (Hoare & Kohane, 2008; Lin & Metters, 2006; Singh, 2006). The polymeric network is able to retain fluids and drastically increase in volume forming a swollen gel phase and, depending on the degree of crosslinking and pH, the network keeps its three-dimensional (3D) structure and will not dissolve (Gupta, Vermani, & Garg, 2002; Kim, Bae, & Okano, 1992).

Fig. 5 shows the water uptake capacity (WUC) of the freeze dried chitosan-6h gels at pH 4.2 and 7.4. All samples absorbed over 400% of their weight in water, regardless of the pH and the concentration of the chitosan-6h. Visual inspection of the samples also showed appreciable volume increase. In addition, no significant difference ($p > 0.05$) was observed in the WUC as function of pH and concentration. The high water uptake by the chitosan-6h gels could be due to their highly porous structure, as indicated by the SEM pictures (Fig. 6).

When chitosan gels are placed in a medium whose pH is below its p*K*a, the amine groups become protonated, which leads to an increase in the osmotic pressure inside the network due to the NH_3^+—NH_3^+ repulsion. In order to equilibrate the osmotic pressure inside and outside the gel, it increases its volume, a process named swelling. In addition, the increase in the gel's total volume can be attributed to the association of hydration water with the NH_3^+ groups, which induces the migration of the anionic counterions from the acid into the gel, resulting in an increase in chemical potential inside of it. This chemical potential is decreased by the migration of water from the outside of the gel, which also results in swelling. The network will continue to imbibe additional water toward infinite dilution. This additional swelling is opposed by the covalent or physical crosslinks, leading to an elastic network. Thus, the gel will reach an equilibrium swelling level. However, if the network chains or crosslinks are degradable, the sample will begin to disintegrate and dissolve, at a rate depending on factors such as pH of the medium and degree of crosslinking (Hoffmann, Seitz, Mencke, Kokott, & Ziegler, 2009).

As shown in Fig. 5, a minimum decrease in weight was observed on the chitosan-6h gel samples during the entire 24 h of experiment, even at pH 4.2, where it was expected that the stability of the Schiff base could have been compromised. However, since the low pH favors the protonation of the amine groups, the repulsion of the $-NH_3^+$ groups might have contributed to a higher degree of swelling. Consequently, at pH 4.2 two process could be occurring concomitantly during the water uptake experiment: the dissolution of the chitosan-6h and an increase in the degree of swelling of the freeze-dried sample. Therefore, the plateau observed in Fig. 5C could be the result of an equilibrium between the increase in weight due to more water uptake and a decrease in weight because of the dissolution of the sample at low pH.

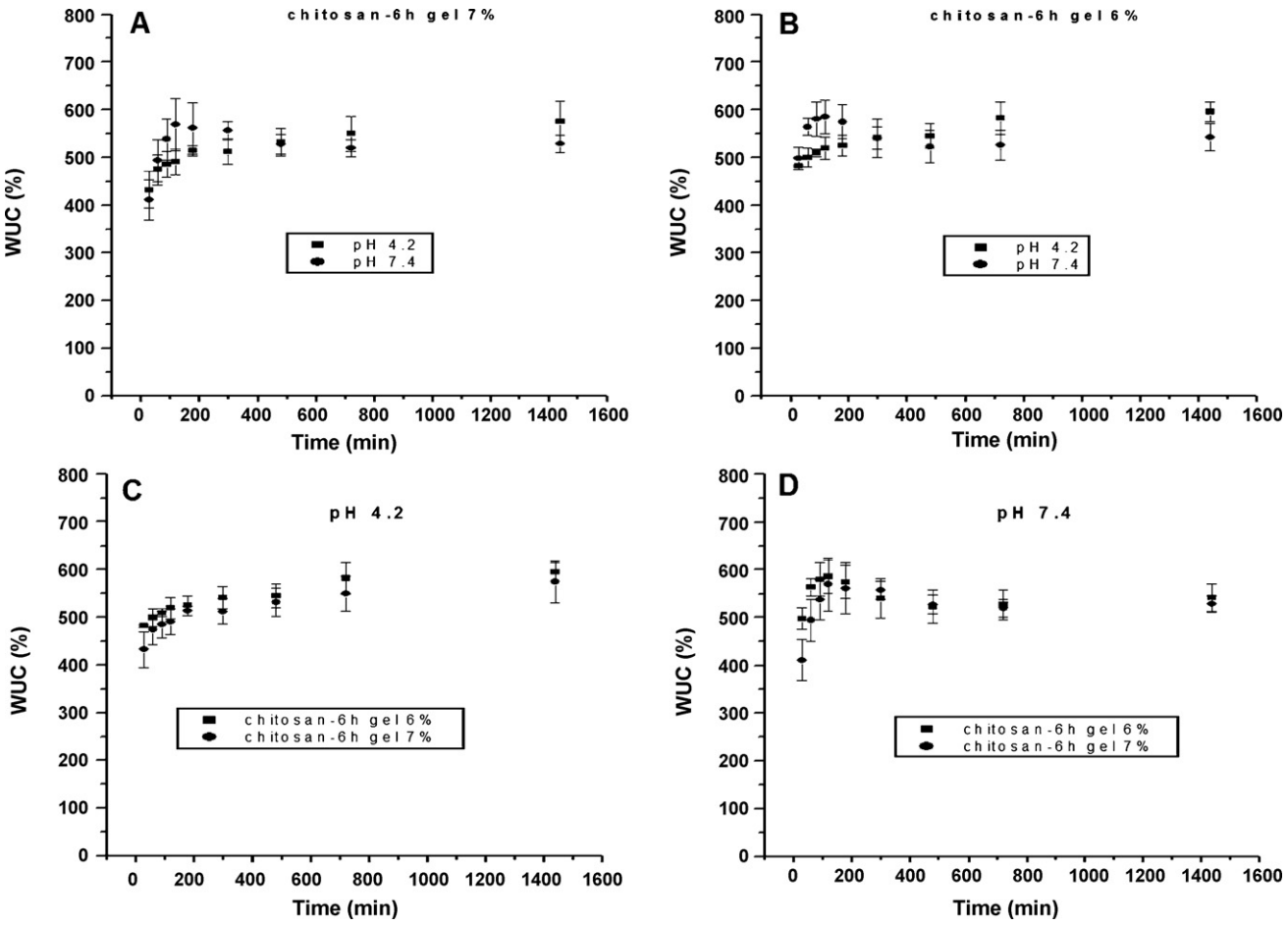

Fig. 5. Effect of pH (A and B) and concentration of chitosan-6h (C and D) on the water uptake capacity (WUC) of chitosan-6h freeze-dried gels ($n = 5$, error bar: standard deviation).

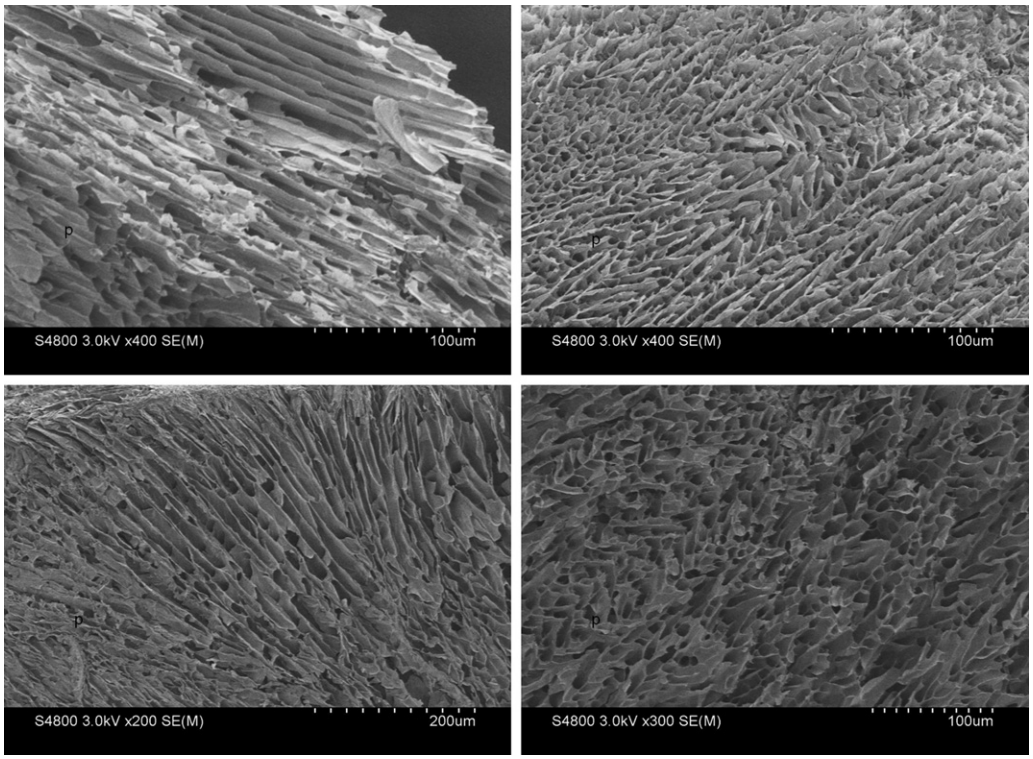

Fig. 6. SEM pictures showing the pore structure of the chitosan-6h 6% freeze-dried gel. All the four pictures were taken from the same sample.

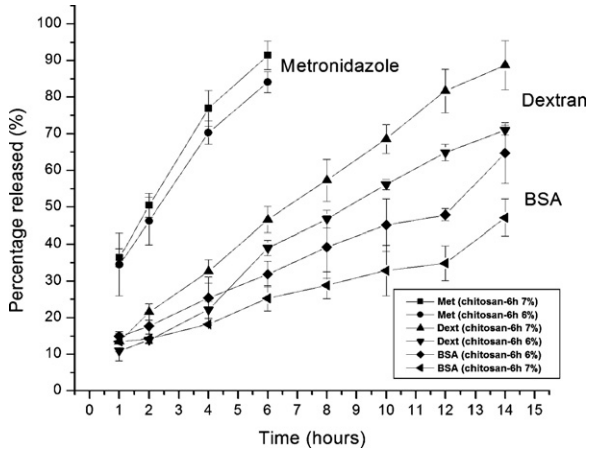

Fig. 7. Percentage released of all model drugs according to the concentration of chitosan-6h in the gel ($n = 3$, error bar: standard deviation). The experiments were performed in phosphate buffer pH 7.4, at 37 °C for 24 h.

3.4. Drug release studies

There are two general methods for loading drugs into gels. In one method, the gel monomer is mixed with the drug, an initiator, with or without a crosslinker, and allowed to polymerize, trapping the drug within the matrix. In another method, a preformed gel or dried gel (hydrogel) is allowed to swell to equilibrium in a drug solution, where the drug diffuses into the gel matrix. The first method has some drawbacks due to the fact that polymerization conditions may have deleterious effects on drug properties. Besides, the removal of the unreacted monomers becomes necessary and this purification step remains challenging (Kim et al., 1992). The second approach to load drugs into gels has also some limitations, mainly due to the incomplete drug loading. In addition, since drug entrapment depends on the extent of swelling and pore size, loading large molecules is sometimes problematic.

In our study, since chitosan-6h is water soluble and the gel forms by dissolving appropriate amounts of this polymer in water, we decided to use water soluble molecules as the model diffusants: metronidazole (Mw 171 Da), FITC-dextran (Mw 40 kDa) and BSA (Mw 66 kDa). First, an aqueous solution of each drug was prepared. Then, chitosan-6h was added to the drug solution under stirring and the gel was prepared *in situ*. Since all three model drugs were previously dissolved in water before adding chitosan-6h, it is assumed that all drugs were homogeneously distributed in the gel. Therefore, some advantages of this method compared to the two aforementioned ones are a more efficient drug loading and a more homogeneous distribution of the diffusant in the gel matrix.

In order to investigate the influence of the chitosan-6h concentration as well as the molecular size of the drugs on their release, experiments were performed at physiological pH (phosphate buffer pH 7.4) using chitosan-6h gels at 6 and 7% (w/w). As shown in Fig. 7, the concentration of chitosan-6h in the gels did not significantly influence ($p > 0.05$) the amount of drug released. Since the rigidity of the gels (represented by G^*) was influenced by the concentration of chitosan-6h (Fig. 4A), a significant change in the extent of drug release from the gels at 6% and 7% of chitosan-6h was expected. For instance (Tang et al., 2007) prepared a thermosensitive chitosan/poly(vinyl alcohol) gel containing BSA. These authors showed that an increase in the chitosan concentration from 0.5% to 1% caused an increase in the strength of the gel (higher G') and this resulted in a slower release of BSA.

However, according to the WUC results of the chitosan-6h gels (Fig. 5), they absorbed more than 400% of their weight in the first 30 min, regardless of the pH of the medium and the concentration of chitosan-6h. Moreover, the highly porous structure of these gels and their apparent low tortuosity indicated by the aligned channels (Fig. 6) might have not offered enough resistance to drug diffusion. Therefore, it is likely to conclude that the increase in the gel strength with the increase in the concentration of chitosan-6h from 6 to 7% was not enough to retard the release of the model molecules used in this study. It is interesting to note that the release of all drugs seems to follow a near zero-order profile.

Theoretically, no solute diffusion is possible within the gel matrix, when mesh size approaches the size of the solute, which means that the release of macromolecules can be sustained from swollen gels due to their significant hydrodynamic radii (Lin & Metters, 2006). Therefore, we hypothesized that the release of larger molecules such as dextran and BSA from the chitosan-6h gels would have an impact on the release. According to Fig. 7, the size of the drug had a significant effect in the diffusion, where the release of metronidazole was faster compared to the larger drugs ($p < 0.05$), which indicates that the increase in the molecule's radius resulted in a slower release.

It is worth mentioning that once the release experiments at pH 7.4 was over, a denser layer was observed on the top of the gels. This layer can be described as a more rigid and dense structure and it was present on top of all samples. A pictorial presentation of this denser layer can be seen on Supplementary Data. A possible explanation for the formation of this layer could be based on the increase of Schiff base formation on the chitosan-6h at pH 7.4.

According to the ^{1}H NMR spectra shown in our previously published work (Azevedo et al., 2012) the appearance of the imine bands (indicative of Schiff base formation) and the concomitant disappearance of the aldehyde bands occurred as the pH increased from 4 to 6, which is where the partial deprotonation of the amine groups occurs. Since the pH of the chitosan-6h 6% is around 5.5, by placing this gel in contact with a higher pH medium would favor the formation of more Schiff base. Because only the top part of the gel was in direct contact with the phosphate buffer solution, the increase in the Schiff base formation initially occurred in the interface between the gel and the buffer. As this solution kept diffusing through the porous structure of the gel, more Schiff base reaction might have occurred and a thicker layer of this denser gel structure was constantly being formed with time.

Therefore, we hypothesized that the formation of this top layer of denser gel might have acted as a barrier for the drug release. In order to corroborate this hypothesis, release experiments were repeated, using BSA as the model diffusant, in two different release media: citrate buffer pH 4.2 and acetate buffer pH 5.5. The former has a pH bellow the pH of the gel (~5.5), while the latter is a very close match with the gel's pH. The results were compared to the release in phosphate buffer pH 7.4. The experiment in pH 4.2 had to be prematurely stopped because the gel dissolved in less than 2 h and a lot of variation on the results was observed (extremely high standard deviation). The fast dissolution of the gel can be explained by the protonation of chitosan-6h and the disruption of the cross-linkages (imine bonds) at low pH. Similar finding was observed by Saito, Hoffman, and Ogawa, 2007 when they developed a new hydrogel system where poly(vinyl amine) (PVAm) was crosslinked with PEG-dialdehyde (OHC-PEG-CHO) by Schiff base. The degradation rates of the swollen PVAm-(OHC-PEG-CHO) hydrogels increased as the pH went down. The authors attributed this result to the poor stability of Schiff bases at lower pHs and the protonation of the amine groups of the PVAm. The authors also reported a much lower degradation of the hydrogels at pH 7.4, where the hydrolysis of the Schiff base was slower (Saito, Hoffman, & Ogawa, 2007).

On the other hand, the water uptake results for the chitosan-6h freeze-dried gels at pH 4.2 (Fig. 5C) show a minimum weight loss

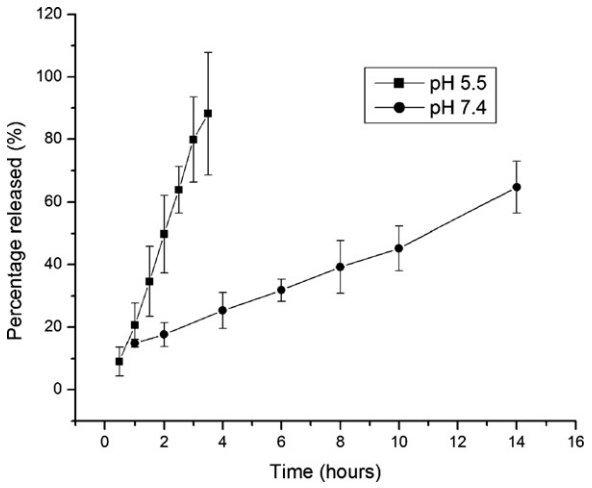

Fig. 8. Release of BSA according to the pH of the release medium: acetate and phosphate buffers, pH 5.5 and 7.4, respectively ($n = 3$, error bar: standard deviation).

during the entire 24 h of experiment. Although the highly porous structure of the freeze-dried gels might have contributed to a fast diffusion of water, the absence of stirring and the time that the samples might have taken to hydrate could be considered as reasonable explanations for the delay in the dissolution of the freeze-dried samples in comparison to the hydrated gels that were used in the release studies.

Fig. 8 shows the release of BSA from chitosan-6h 6% gels in acetate and phosphate buffer solutions. As expected, neither the dissolution of the gels nor the formation of the denser layer on top of the samples was observed in acetate buffer solution (pH 5.5). The fact that the pH of the medium was similar to that of the gels might have not changed the extent of crosslinkage. Thus, the release of BSA was faster at this pH when compared to that at pH 7.4, which suggests that the formation of the denser layer at higher pH acted as a barrier for the drug release. Another possible explanation for the slower release of BSA at higher pH could be based on the change in the extent of electrostatic interaction between chitosan-6h matrix and BSA. The isoelectric point of BSA is 4.7 and at pH > pI BSA is negatively charged (Wang, Huang, Jiang, & Tang, 2012). Although the charge density of BSA must have increased when the pH went from 5.5 to 7.4, chitosan-6h becomes deprotonated at pH 7.4 (chitosan p*K*a is ~6.5), which suggests less electrostatic interaction between chitosan-6h and BSA at this pH. In addition, the increase in Schiff base formation with the increase in pH, as shown by the pH-dependent ^{1}H NMR spectra in our previous work (Azevedo et al., 2012), corroborates the hypothesis of increase in crosslinking density with an increase in pH.

The reasonable linearity of both curves indicates that the near zero-order profile remains regardless of the pH of the medium.

4. Conclusions

The rheological properties of the self-crosslinked gels were influenced by the concentration of the chitosan-6h, where higher concentration originated more rigid gels. The water uptake capacity of the freeze-dried gels was above 400% of its dry weight and neither the chitosan-6h concentration nor the pH of the medium influenced the results. As a drug carrier, the chitosan-6h gels released the model molecules following a near zero-order profile and the concentration of chitosan-6h in the gel did not influence the amount of solute released. On the other hand, the pH of the medium and the size of the model molecule influenced the release, where the amount released decreased as the pH went over the gel's pH, which was explained by a possible increase in the Schiff base formation at higher pH.

Acknowledgement

The authors would like to thank CAPES/Fulbright exchange program for financial support.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.carbpol.2012.06.017.

References

Azevedo, E. P., Santhana Mariappan, S. V., & Kumar, V. (2012). Preparation and characterization of chitosans carrying aldehyde functions generated by nitrogen oxides. *Carbohydrate Polymers*, *87*, 1925–1932.

Berger, J., Reist, M., Mayer, J. M., Felt, O., & Gurny, R. (2004). Structure and interactions in chitosan hydrogels formed by complexation or aggregation for biomedical applications. *European Journal of Pharmaceutics and Biopharmaceutics*, *57*

de Morais, W. A., Pereira, M. R., & Fonseca, J. L. C. (2012). Characterization of gelification of chitosan solutions by dynamic light scattering. *Carbohydrate Polymers*, *87*.

Deem, D. E. (1988). Rheology of dispersed systems. In H. A. Lieberman, M. M. Rieger, & G. S. Banker (Eds.), *Pharmaceutical dosage forms: Disperse systems* (pp. 367–425). New York: Marcel Dekker, Inc.

Gupta, P., Vermani, K., & Garg, S. (2002). Hydrogels: From controlled release to pH-responsive drug delivery. *Drug Discovery Today*, *7*.

Hoare, T. R., & Kohane, D. S. (2008). Hydrogels in drug delivery: Progress and challenges. *Polymer*, *49*.

Hoffmann, B., Seitz, D., Mencke, A., Kokott, A., & Ziegler, G. (2009). Glutaraldehyde and oxidised dextran as crosslinker reagents for chitosan-based scaffolds for cartilage tissue engineering. *Journal of Materials Science. Materials in Medicine*, *20*.

Kim, S. W., Bae, Y. H., & Okano, T. (1992). Hydrogels: Swelling, drug loading, and release. *Pharmaceutical Research*, *9*.

Kumar, V., & Yang, T. (2002). HNO_3/H_3PO_4—$NaNO_2$ mediated oxidation of cellulose – preparation and characterization of bioabsorbable oxidized celluloses in high yields and with different levels of oxidation. *Carbohydrate Polymers*, *48*.

Lavertu, M., Xia, Z., Serreqi, A. N., Berrada, M., Rodrigues, A., Wang, D., et al. (2003). A validated ^{1}H NMR method for the determination of the degree of deacetylation of chitosan. *Journal of Pharmaceutical and Biomedical Analysis*, *32*.

Lin, C. C., & Metters, A. T. (2006). Hydrogels in controlled release formulations: Network design and mathematical modeling. *Advanced Drug Delivery Reviews*, *58*.

Muzzarelli, R. A. A. (2009). Genipin-crosslinked chitosan hydrogels as biomedical and pharmaceutical aids. *Carbohydrate Polymers*, *77*.

Muzzarelli, R. A. A., Boudrant, J., Meyer, D., Manno, N., DeMarchis, M., & Paoletti, M. G. (2012). Current views on fungal chitin/chitosan, human chitinases, food preservation, glucans, pectins and inulin: A tribute to Henri Braconnot, precursor of the carbohydrate polymers science, on the chitin bicentennial. *Carbohydrate Polymers*, *87*.

Prabaharan, M. (2008). Review paper: Chitosan derivatives as promising materials for controlled drug delivery. *Journal of Biomaterials Applications*, *23*.

Ravi Kumar, M. N. V. (2000). A review of chitin and chitosan applications. *Reactive and Functional Polymers*, *46*, 1–27.

Ravi Kumar, M. N. V., Muzzarelli, R. A. A., Muzzarelli, C., Sashiwa, H., & Domb, A. J. (2005). Chitosan chemistry and pharmaceutical perspectives. *ChemInformatics*, *36*.

Rinaudo, M. (2006). Chitin and chitosan: Properties and applications. *Progress in Polymer Science*, *31*.

Sahiner, N., Singh, M., Kee, D. D., John, V. T., & McPherson, G. L. (2006). Rheological characterization of a charged cationic hydrogel network across the gelation boundary. *Polymer*, *47*.

Saito, H., Hoffman, A. S., & Ogawa, H. I. (2007). Delivery of doxorubicin from biodegradable PEG hydrogels having Schiff base linkages. *Journal of Bioactive and Compatible Polymers*, *22*.

Schott, H. (2000). Rheology. In *Anonymous remington: The science and practice of pharmacy*. Baltimore, MD: Lippincott Williams & Wilkins., pp. 335–355.

Singh, A. (2006). External stimuli response on a novel chitosan hydrogel crosslinked with formaldehyde. *Bulletin of Materials Science*, *29*, 233.

Tang, Y., Du, Y., Hu, X., Shi, X., & Kennedy, J. F. (2007). Rheological characterisation of a novel thermosensitive chitosan/poly(vinyl alcohol) blend hydrogel. *Carbohydrate Polymers*, *67*.

Wang, F., Huang, W., Jiang, L., & Tang, B. (2012). Quantitative determination of proteins based on strong fluorescence enhancement in curcumin–chitosan–proteins system. *Journal of Fluorescence*, *22*.