



Xanthan hydrogel films: Molecular conformation, charge density and protein carriers



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ARTICLE INFO

Article history:

Received 11 June 2013

Received in revised form 30 August 2013

Accepted 13 October 2013

Available online 22 October 2013

Keywords:

Xanthan hydrogels films

Molecular conformation

Circular dichroism

Bovine serum albumin

Lysozyme

Bactericidal activity

ABSTRACT

In this article the molecular conformation of xanthan chains in hydrogel films was investigated by means of circular dichroism, showing substantial differences between xanthan hydrogel prepared in the absence (XNT) and in the presence of citric acid (XCA). The xanthan chains in XNT hydrogels films presented ordered conformation (helixes), while in XCA they were in the disordered conformation (coils), exposing a larger number of carboxylate groups than XNT. The large charge density in XCA hydrogels was evidenced by their behavior under variable ionic strength. Studies about the application of XNT and XCA for loading and delivering of bovine serum albumin (BSA) and lysozyme (LYZ) showed that both events are controlled by hydrogels and proteins net charge, which can be triggered by pH. The preservation of LYZ native conformation after hydrogel loading explained the substantial bactericidal activity of LYZ loaded hydrogels and enables their use as active wound dressings.

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1. Introduction

Hydrogels, tridimensional networks of hydrophilic polymers, have been used as biomaterials for the creation of artificial muscles (Jilie & Li, 2007), carriers for drug or proteins delivery (Fogaça & Catalani, 2013) and biological micro-electromechanical systems, the Bio-MEMS (Bashir, 2004) due to their interesting characteristics as biocompatibility, softness and ability to swell in water or biological fluids (Saraydin & Çaldıran, 2001). Depending on the polymer characteristics, hydrogels can reversibly swell and shrink upon changes in environmental conditions, such as temperature, pH (Bueno, Bentini, Catalani, & Petri, 2013; Thakur, Wanchoo, & Singh, 2011), electric field, light, pressure, ionic strength (Lai & Li, 2010) and solvent (Jilie & Li, 2007). Such stimuli responsive hydrogels are interesting for applications as controlled drug delivery (Fajardo et al., 2013; Jilie & Li, 2007; Soppimath, Aminabhavi, Dave, Kumbar, & Rudzinski, 2002).

Polysaccharides based hydrogels are of great interest mainly for biomedical applications particularly due to their biocompatibility and similarity with biological systems. Xanthan gum is a branched and high molecular weight polysaccharide with acidic characteristic predominantly produced by *Xanthomonas campestris* (Geremia & Rinaudo, 2005). It is largely used as thickener agent in food, cosmetics and drilling fluids (Geremia & Rinaudo, 2005; Petri &

Queiroz Neto, 2010; Queiroz Neto, Biscaia, & Petri, 2007). It is composed by D-glucosyl, D-mannosyl, and D-glucuronyl acid residues in a 2:2:1 molar ratio and variable proportions of O-acetyl and pyruvyl residues. Side-chains consist on a trisaccharide composed of mannose (β-1,4) glucuronic acid (β-1,2) mannose, attached to alternate glucose residues in the backbone by α-1,3 linkages. The deprotonation of O-acetyl and pyruvyl residues at pH > 4.5 increases negative charge density along the xanthan chains, enabling their physical crosslinking mediated by Ca²⁺ ions (Bergmann, Furth, & Mayer, 2008; Dário, Hortêncio, Sierakowski, Queiroz Neto, & Petri, 2011). Xanthan can also be chemically crosslinked by using adipic acid dihydrazide (Bejenariu, Popa, Picton, & Le Cerf, 2009; Bejenariu, Popa, Dulong, Picton, & Le Cerf, 2009), sodium trimetaphosphate (STMP) (Bejenariu, Popa, Picton, et al., 2009; Bejenariu, Popa, Dulong, et al., 2009) and citric acid, a non-toxic crosslinker for polysaccharides (Bueno et al., 2013; Reddy & Yang, 2010; Yang, Wang, & Kang, 1997).

Chemical hydrogels differ from physical hydrogels; in the former the polymer chains are crosslinked by covalent bonds, while in the latter the chains are only physically crosslinked. Recently the synthesis and characterization of xanthan (XNT) and xanthan-citric acid (XCA) chemical hydrogels were reported (Bueno et al., 2013). In the absence of citric acid (XNT) the intra- and intermolecular ester bonds are due to xanthan chains dehydration, limiting the crosslinking density. XCA presented higher crosslinking density and lower swelling degree than XNT due to the many crosslinkings between citric acid and xanthan pyruvyl, acetyl and OH groups. The higher crosslinking density in XCA turned them more rigid than

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XNT, as evidenced by tensile tests. The diffusion mechanisms of water into XNT and XCA were determined by means of tensiometry; initially the diffusion was stereoselective, controlled by wicking properties, and then it changed to Fickian (XNT) or anomalous (XCA) behavior. XNT and XCA hydrogels presented stability under acidic or neutral conditions, but the swelling degree increases about four times under alkaline conditions due to the partial hydrolysis of ester bonds; such behavior enables their use as drug enteric vehicles.

The order disorder transition of xanthan chains is controlled by temperature and ionic strength. At low ionic strength or high temperature chains assume coil conformation with persistence length of ~ 5.0 nm (Tinland & Rinaudo, 1989). On the other hand, under high ionic strength or low temperature, xanthan chains are arranged in helical conformation with persistent length ~ 35.0 nm (Tinland & Rinaudo, 1989). Such transitions can be detected by rheology (Renaud, Belgacem, & Rinaudo, 2005) or circular dichroism (CD) (Goycoolea, Milas, & Rinaudo, 2001). Although CD has been often used for macromolecules in solution or self-assembled (Nochi et al., 2010; Turgeon, Schmitt, & Sanchez, 2007), it has been seldom applied to investigate the conformation of crosslinked chains in swollen films. In the present work, the molecular conformations of xanthan chains in the XNT and XCA chemical hydrogels films were investigated by means of CD under variable ionic strength. Moreover, the ability of XNT and XCA to load and release two proteins, namely, bovine serum albumin (BSA) and lysozyme (LYZ) at different pH values was studied. BSA and lysozyme (LYZ) were chosen, because they are globular proteins with distinct isoelectric points (pI); LYZ and BSA pIs are 10.7 (Muszanska, Busscher, Herrmann, van der Mei, & Norde, 2011) and 4.8 (Serefoglou, Oberdisse, & Staikos, 2007), respectively. So, at physiological pH (7.4), LYZ is positively and BSA is negatively charged. These characteristics allow evaluating the effect of charges on the interaction with the polymer network. Moreover, BSA, one of the most abundant proteins, is widely used in diagnosis tests (Carter & Ho, 1994) and as shielding agents to avoid non-specific adsorption, while LYZ, is an enzyme with bactericidal activity (Bengani, Leclerc, & Chauhan, 2012). The biocidal activity of LYZ loaded-hydrogels was determined in order to evaluate their potential as bactericidal wound dressings.

2. Experimental

2.1. Materials

Commercial xanthan (Kelzan[®], CP Kelco, USA, degree of pyruvate = 0.38, degree of acetyl = 0.41, $M_v \sim 1.0 \times 10^6$ g/mol, degree of polymerization ~ 1072) was used as received. Citric acid (Analitica Quimica, Brazil) was recrystallized twice from water prior to use. Analytical grade NaCl, HCl NaHCO₃ and Na₂CO₃ were purchased from Casa Americana, Brazil. Trizma[®] Base, bovine serum albumin (BSA, A3803, 98% purity) and lysozyme (LYZ, L7651, 95% purity) were provided by Sigma–Aldrich. Deionized (Milli-Q) water was used in all experiments.

2.2. Hydrogels films preparation

All xanthan-based hydrogels were produced by casting a 6 g L⁻¹ xanthan aqueous solution in the absence or in the presence of citric acid at 0.3 g L⁻¹, at 45 °C overnight, as described elsewhere (Bueno et al., 2013). The solutions were homogenized with an Ika Turrax[®] stirrer at 18,000 rpm for 3 min and submitted to centrifugation for 5 min at 3600 rpm, to remove air bubbles prior to casting. Crosslinking was achieved by heating the dried films (~ 0.02 mm thick) at 165 °C for 7 min. The sol fraction was extracted with water at ~ 70 °C, under gentle magnetic stirring, during 24 h. The resulting

xanthan (XNT) and xanthan-citric acid (XC) hydrogels films were dried at 45 °C for 48 h.

2.3. Circular dichroism spectroscopy

Circular dichroism (CD) measurements were performed on a Jasco J-720 spectropolarimeter, in the wavelength range of 190–260 nm, at (25 ± 1) °C, using a 0.5 cm quartz cell. CD spectra of xanthan solutions at 2.0 g L⁻¹ or 6.0 g L⁻¹ were collected at pH 6.5 using scan speed of 50 nm min⁻¹, time constant of 0.5 s and bandwidth of 1 nm. Eight scans were accumulated for each sample.

In the case of swollen XNT (~ 0.8 mm thick) and XCA (~ 0.4 mm thick) hydrogels films (or the correspondent LYZ-containing hydrogel), they were cut in a rectangular format (0.5 cm $\times$ 1.5 cm) and carefully stretched on the external quartz cell wall (normal incidence), as shown in Supplementary Material, Figure S1. CD spectra were collected at the same conditions described above. In order to evaluate the effect of ionic strength on the molecular conformation of xanthan chains in the XNT and XCA hydrogels, samples swollen in water (pH 6.5) were immersed in 0.001, 0.01, 0.1 and 1.0 mol L⁻¹ NaCl for 30 min prior to the measurements.

2.4. LYZ and BSA loading to hydrogels films

Dried XCA and XNT hydrogels films (~ 4.5 mg each) were swollen with 450 μ L of LYZ or BSA aqueous solutions (10 g L⁻¹, pH 6.5) for 48 h, at (25 ± 1) °C. One should notice that the medium ionic strength was very low, because proteins have very low impurity contents, as stated by provider. After swelling, films were quickly rinsed with distilled water and carefully dried with absorbent paper to remove water excess (Supplementary Material, Figure S2A) and then they were freeze-dried. The amount of loaded protein was assessed by the sulfur concentration in the samples, determined by inductively coupled plasma (ICP)–atomic emission spectrometry (Spectro Circus CCD apparatus). Controls composed by hydrogels swollen with pure water were also produced and analyzed as blanks.

2.5. LYZ and BSA release from hydrogels films

Dried XCA and XNT samples (~ 4.5 mg) were swollen with 450 μ L of LYZ or BSA aqueous solutions (10 g L⁻¹) for 48 h, at (25 ± 1) °C. After swelling, samples were quickly rinsed with distilled water and carefully dried with absorbent paper to remove water excess. Protein loaded hydrogels were immersed into 3 mL of media at different pH values, namely, pH 2.0 (HCl 0.010 mol L⁻¹/NaCl 0.010 mol L⁻¹), pH 4.8 (CH₃COOH/CH₃COONa, 0.050 mol L⁻¹), pH 7.0 (Tris/HCl, 0.050 mol L⁻¹) and pH 10.0 (NaHCO₃/Na₂CO₃, 0.050 mol L⁻¹). After each hour, 1.0 mL was collected, and replaced by 1.0 mL of the corresponding medium (Supplementary Material, Figure S2B). The protein concentration in the collected aliquots was determined by UV–vis spectrophotometry (at 280 nm), using a calibration curve (Shimadzu Multispec-1501) (Supplementary Material, Figure S3).

The diffusion mechanism of BSA and LYZ from the XNT and XCA hydrogels films was evaluated by the diffusional coefficient, n , which can be calculated according to (Peppas, 1986):

$$\frac{M_t}{M_e} = kt^n \quad (1)$$

where M_t and M_e are the masses of released protein at a given time t and at equilibrium condition, respectively, k is a constant and n is the diffusional coefficient. The linear coefficient of $\ln(M_t/M_e)$ as a function of $\ln(t)$ corresponds to n . Typically the time range t used in Eq. (1) was from 15 to 60 min.

2.6. Evaluation of bactericidal activity of LYZ loaded hydrogels films

Bactericidal activity of LYZ-hydrogel films were evaluated according to a conventional protocol (Weisner, 1984). First the turbidity (τ_i) of aqueous dispersions (pH 5.7) of *Micrococcus luteus* (ATCC 4698), a Gram-positive bacterium, at 2 g L^{-1} was measured at $(25 \pm 1)^\circ\text{C}$ and 546 nm, using a Beckmann Coulter DU-600 spectrophotometer. Then swollen LYZ loaded hydrogels films were immersed into 5 mL of *M. luteus* suspension. Suspensions of *M. luteus* at 2 g L^{-1} in the absence and in the presence of free LYZ 1.3 g L^{-1} were used as positive and negative controls, respectively. XCA and XNT hydrogels films without LYZ were also tested as references. After 30 min of incubation at 25°C , the turbidity of suspensions (τ_f) was measured by spectrophotometry at 546 nm. These conditions were chosen based on previous studies (Amim, Maia, Miranda, Urzúa, & Petri, 2012; Dário, de Paula, Paula, Feitosa, & Petri, 2010; Silva, Urzúa, & Petri, 2009). Bacteria disruption indicated antimicrobial activity and was correlated with the relative decrease of turbidity ($\Delta\tau$); the largest is $\Delta\tau$, the more efficient is the antimicrobial agent:

$$\Delta\tau(\%) = \frac{\tau_i - \tau_f}{\tau_i} \times 100 \quad (2)$$

3. Results and discussion

3.1. Molecular conformations of xanthan chains in the XNT and XCA swollen hydrogels films

In the dilute range ($<2.5 \text{ g L}^{-1}$), free xanthan chains might undergo conformational changes from helix to random coils depending on the medium ionic strength, temperature and pH. Disordered and flexible chains structures appear under low ionic strength or high temperature (Geremia & Rinaudo, 2005; Tinland & Rinaudo, 1989). At low temperature ($<44^\circ\text{C}$) (Gravanis, Milas, Rinaudo, & Tinland, 1987) or high ionic strength xanthan chains present very ordered structures, as single or double helix conformations (Geremia & Rinaudo, 2005). Upon increasing medium pH other conformational changes from double helical to coils were detected by light scattering (Bejenariu, Popa, Picton, & Le Cerf, 2010), rheological measurements (Gravanis et al., 1987) and circular dichroism (Bejenariu et al., 2010; Bindal, Narsimhan, Hem, & Kulshreshtha, 2007; Matsuda, Biyajima, & Sato, 2009; Milas & Rinaudo, 1979; Rinaudo, Milas, Bresolin, & Ganter, 1999).

CD spectroscopy is a powerful tool to determine the molecular conformations of polysaccharides in solution (Fasman, 1996). The carboxylic or carboxylate groups of the D-glucuronic acid and the pyruvate groups that are present in the xanthan side chains are optically active, presenting a maximum of absorbance at $\sim 202 \text{ nm}$, which corresponds to the $n \rightarrow \pi^*$ transition, and a minimum at $\sim 220 \text{ nm}$, due to acetate function (Bejenariu et al., 2010). The decrease of ellipticity in the range of 200–210 nm indicates a decrease in the helix conformation. The increase of ellipticity in the range between 220 nm and 250 nm indicates an increase of coil conformation (Bindal et al., 2007), denaturation of xanthan chains (Matsuda et al., 2009) or a disordered state of xanthan chains (Rinaudo et al., 1999). One novelty of this study is the investigation of molecular conformations of crosslinked xanthan chains in swollen hydrogel films. Fig. 1 shows CD spectra obtained at pH 6.5 for swollen XNT and XCA hydrogels. The helix conformation of xanthan chains observed in solution is indicated by the positive band at $\sim 202 \text{ nm}$ and the negative band at $\sim 220 \text{ nm}$ and is retained after crosslinking in the absence of citric acid (swollen XNT hydrogels). On the other hand, crosslinking in the presence of citric acid (swollen XCA hydrogels) led to coil conformation

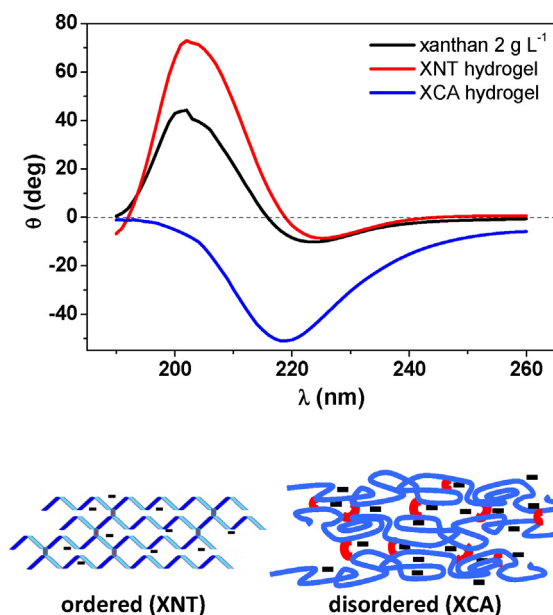


Fig. 1. CD spectra of xanthan chains in solution (2 g L^{-1} , black line), XNT (red line) and XCA (blue line) films swollen in water, along with the schematic representation of ordered and disordered states of crosslinked chains. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

and denaturation of crosslinked xanthan chains, similar to coil conformation of xanthan chains in solution (Matsuda et al., 2009). Such structural differences might be explained by three factors. The first one is that the presence of citric acid in xanthan solutions used to produce the hydrogels causes a small decrease in the ellipticity. This hypothesis was checked with CD measurements performed for those xanthan solutions with citric acid before crosslinking. In fact, CD spectra obtained under these conditions (Supplementary Material, Figure S4A) evidenced a small decrease in the ellipticity in comparison to those determined for pure xanthan solutions. The second one is the fact that XCA hydrogels have higher crosslinking density than XNT hydrogel, as indicated by their swelling capacities (Bueno et al., 2013) and the third one is the higher density of negative charges in XCA than in XNT after crosslinking. In order to gain insight about the electrostatic effects in the conformational state of crosslinked xanthan chains, CD measurements were performed with XNT and XCA swollen in NaCl solutions with increasing concentrations. Fig. 2 shows the effect of ionic strength on the molecular conformation of swollen XNT and XCA hydrogels. For comparison, CD spectra of xanthan solution at 2 g L^{-1} as a function of ionic strength are provided as Supplementary Material (Supplementary Material, Figure S4B). Xanthan chains in solution and crosslinked in the absence of citric acid (XNT hydrogel) presented similar behavior. The increase in the ionic strength had no significant effect of the ordered state, except at 1.0 mol L^{-1} NaCl, which caused a decrease in the ellipticity and a shift from ~ 202 – 208 nm , indicating partial destruction of helix conformation. Xanthan chains in the XCA hydrogels films are more sensitive to ionic strength effect than xanthan chains in solution or in the XNT hydrogel films. Upon increasing the NaCl concentration from 0.001 mol L^{-1} to 0.100 mol L^{-1} the intensity of the minimum at 220 nm decreased, a shift to lower wavelength range was observed and a maximum at $\sim 202 \text{ nm}$ appeared. All these features indicate a disorder order transition of crosslinked chains, caused by screening of negative charges of xanthan chains in the XCA. Interestingly, at 0.100 mol L^{-1} NaCl the CD spectra for xanthan chains in solution, XNT and XCA hydrogel presented similar ordered conformation (Fig. 2C). However, at 1.0 mol L^{-1} NaCl,

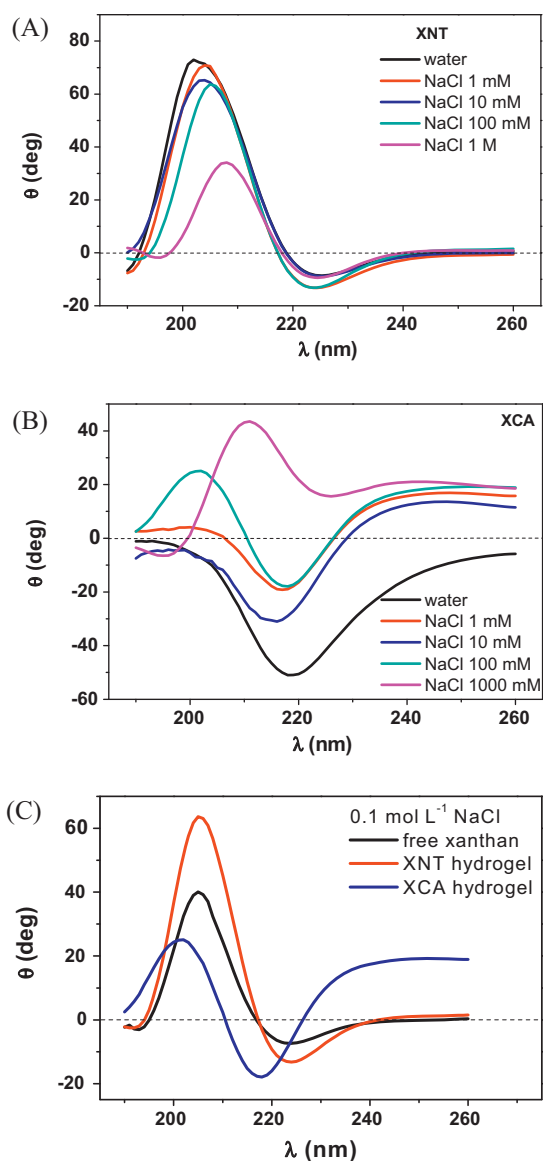


Fig. 2. CD spectra of (A) swollen XNT films, (B) swollen XCA films as a function of ionic strength, (C) free xanthan at 2 g L⁻¹, XNT and XCA swollen films at 0.1 mol L⁻¹ NaCl.

the xanthan chains in the XCA hydrogels presented a positive band at ~ 211 nm and the negative band disappeared, indicating that helix conformation was perturbed by the excess of salt and visually the XCA hydrogels turned turbid, indicating reduced solubility. Thus, the CD spectra obtained for XCA and XNT hydrogels films as a function of ionic strength allow concluding that the XCA hydrogels have higher charge density than XNT hydrogels and this characteristic, summed to the higher crosslinking density, might explain the distinct xanthan chains conformation in each hydrogel film.

3.2. BSA loading and release

Hydrogels films were swollen with BSA solutions at 10 g L⁻¹ and pH 6.5. After drying, samples were analyzed by ICP-AES, to quantify sulfur content. Sulfur amount on hydrogel can be used to estimate the amount of adsorbed protein. BSA molecule contains in its structure 39 aminoacids with one sulfur atom each one: 4 units of methionine and 35 units of cysteine. Sulfur atoms correspond to 1.9% of BSA molecular weight.

Table 1

Diffusional coefficient n and constant k calculated for BSA and LYZ release from XNT and XCA hydrogels films at different pHs in the time range from 15 min to 60 min.

pH	Hydrogel	n		k	
		BSA	LYZ	BSA	LYZ
2.0	XNT	0.39 ± 0.08	0.6 ± 0.1	1.0 ± 0.2	0.08 ± 0.01
	XCA	^a	0.5 ± 0.1	^a	0.15 ± 0.03
4.8	XNT	^a	—	^a	—
	XCA	^a	—	^a	—
7.0	XNT	1.2 ± 0.2	0.46 ± 0.07	1.6 ± 0.3	0.10 ± 0.02
	XCA	^a	0.45 ± 0.01	^a	0.10 ± 0.01
10.0	XNT	1.6 ± 0.4	0.98 ± 0.09	2.4 ± 0.6	0.010 ± 0.002
	XCA	^a	0.83 ± 0.08	^a	0.020 ± 0.002

^a Too large standard deviation values.

The amount of BSA loaded in the XNT hydrogels films was larger than in the XCA hydrogels films, namely, (0.30 ± 0.01) g_{BSA}/g_{XNT} and (0.04 ± 0.01) g_{BSA}/g_{XCA}, respectively. The effect of ionic strength on the CD spectra in Fig. 2 revealed that XCA hydrogels have higher charge density than XNT, which stems from carboxylate groups. At pH 6.5 BSA has negative net charge. Thus the electrostatic repulsion between XCA and BSA explains the very low BSA loading in the XCA hydrogels. In the case of XNT the adsorbed amount is higher because (i) most carboxylate groups originally present in the xanthan structure were used in the esterification reaction among the chains (crosslinking), (ii) the swelling degree is higher (crosslinking density is lower) and (iii) the ordered conformation of xanthan chains in the XNT hydrogels avoids exposition of xanthan charges to the medium.

Fig. 3 shows the BSA release from XNT and XCA hydrogels films as a function of pH and time. After 1 h the BSA release from XNT was complete at pH 2.0, 7.0 and 10.0. However, at pH 4.8, which is close to the BSA pI, the BSA release from XNT or XCA was limited to 15%. The pK_a of xanthan carboxylic acid groups is close to 4.6. Thus at pH 2 xanthan chains are uncharged and BSA molecules are positively charged; strong interaction between hydrogel and BSA is not expected. At pH 4.8, near the isoelectric point of BSA (4.9), the release is hindered because BSA solubility is reduced and BSA positively charged patches can bind to xanthan carboxylate groups. Similar behavior was observed for oxidized galactomannans (Sierakowski, Freitas, Fujimoto, & Petri, 2002) carboxymethylcellulose (Fujimoto, Reis, Petri, & Campana Filho, 2002), oxidized xyloglucans (Jo, Petri, Valenga, Lucyszyn, & Sierakowski, 2009) and polycations (Silva, Urzúa, Petri, & Dubin, 2010). At pH 7 xanthan and BSA have negative net charge and release is favored by electrostatic repulsion. Similar behavior was observed for the release of BSA from methacrylated dextran hydrogels (Schillemans, Hennink, & van Nostrum, 2010). In basic medium, partial hydrolysis of ester groups that form crosslinks takes place (Bueno et al., 2013), facilitating the protein release. Diffusional coefficients (n) and constant k values, calculated from linearization of release curves obtained for BSA from XNT (Supplementary Material, Figure S5) in the time range of 15–60 min, are presented in Table 1. Diffusional coefficients for BSA release from XNT at all pH values were calculated, but at pH 4.8 the standard deviation was too high (in some cases, >100%), mainly because the amounts of protein loaded or released were too low. A pH 2, the n value of 0.39 ± 0.08 for the release of BSA from XNT indicated a Fickian Diffusion (typical n value is 0.5) mechanism, which describes the transport of molecules through a matrix without any specific interactions between solute and matrix. At pH 7 or pH 10 the n values are 1.2 ± 0.2 and 1.6 ± 0.4 , indicating the Super-Case II Diffusion ($n > 1.0$), or very fast release mainly due to electrostatic repulsion (Schillemans et al., 2010), large pores and low crosslinking density of XNT (Bueno et al., 2013), which facilitate BSA release. The release of BSA molecules from XCA as a

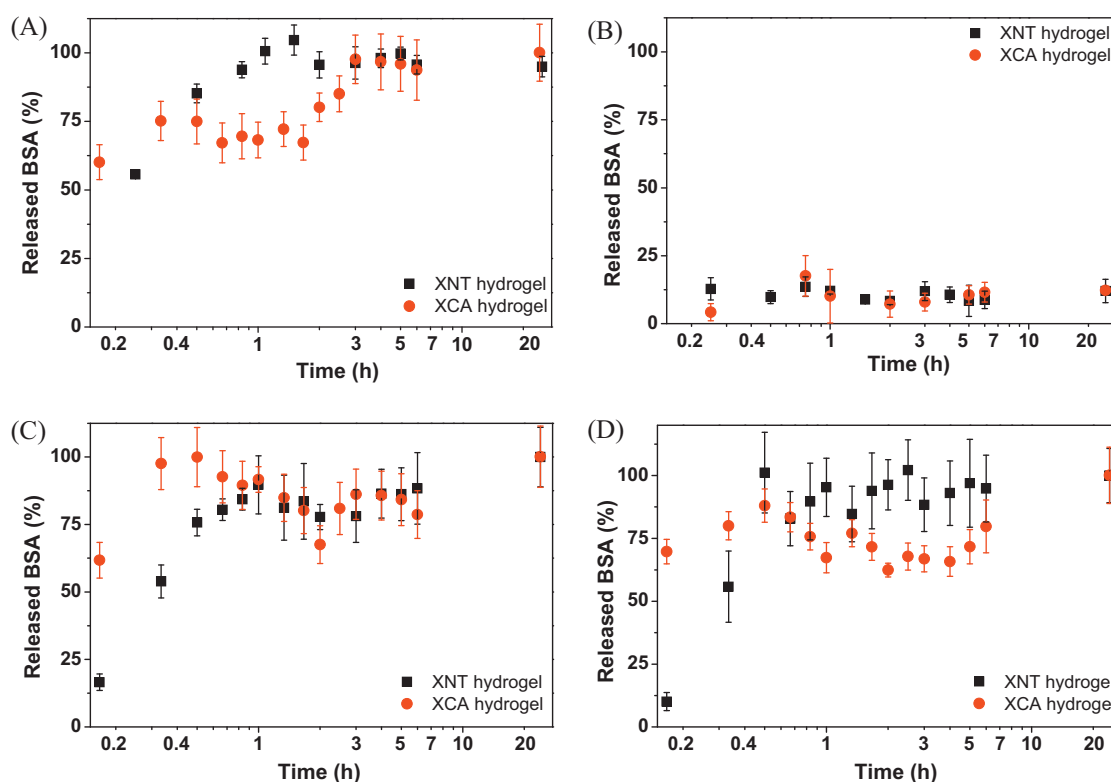


Fig. 3. Cumulative release of BSA from XNT (black square) and XCA (red circle) hydrogels at (A) pH 2.0, (B) pH 4.8, (C) pH 7.0 and (D) pH 10.0 as a function of time. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

function of time could not be fitted to Eq. (1); either because the initial release was already very high or because the changes in the released amount were within the standard deviation values.

3.3. LYZ loading and release

XNT and XCA films were swollen in LYZ solutions at 10 g L^{-1} and pH 6.5. After drying, samples were analyzed by ICP-AES, to quantify sulfur content. LYZ molecule contains in its structure 10 aminoacids with one sulfur atom each one: 2 units of methionine and 8 units of cysteine. Sulfur atoms correspond to 2.3% of LYZ molecular weight. The adsorbed amounts of LYZ were $(0.33 \pm 0.01) \text{ g}_{\text{BSA}}/\text{g}_{\text{XNT}}$ and $(0.24 \pm 0.07) \text{ g}_{\text{BSA}}/\text{g}_{\text{XCA}}$ for XNT and XCA, respectively. It was larger for XNT than for XCA hydrogels because XNT hydrogels present larger pores, lower crosslinking density and higher swelling degree (Bueno et al., 2013). At pH 6.5, the xanthan chains in XNT or XCA are negatively charged and LYZ has positive net charge ($pI \sim 11.35$), favoring electrostatic interaction between them. A similar behavior was observed for the adsorption of LYZ onto negatively charged surfaces, such as, poly(NIPAM-co-acrylic acid) (Johansson, Gernandt, Bradley, Vincent, & Hansson, 2010) or *Sterculia striate* polysaccharide (Dário et al., 2010).

LYZ release from XNT and XCA hydrogels films as a function of time and pH is presented in Fig. 4. At pH 2.0 (Fig. 4A), regardless the hydrogel type, after 1 h the release of LYZ was complete. Considering pI value of LYZ, in acid conditions, hydrogel carboxylate groups are protonated and LYZ is positive. Thus, practically no electrostatic interaction occurs between hydrogel and LYZ to keep the enzymes bound to the polymer chains. On the other hand, at pH 7.0 (Fig. 4A), only 10% and 20% of the initial loading was released from XCA and XNT, respectively. The electrostatic interaction between polymeric network and enzyme prevented the release. Comparing both hydrogels, LYZ release from XCA hydrogels films was lower because its negative charge density is higher and the negative charges are

more exposed to the medium due to the xanthan chains disordered conformation, as evidenced by CD spectroscopy. At pH 10.0 (Fig. 4B), regardless the hydrogel type, the LYZ release was slower; after 24 h the maximal release was $\sim 80\%$ of initial loading. Under such conditions, hydrolysis of crosslinking favored the LYZ release.

Diffusional coefficients (n) and k values calculated from linearization of LYZ release curves from both hydrogels (Supplementary material, Figure S6) are presented in Table 1. At pH 2.0 and pH 7.0, n values indicated Fickian behavior for both hydrogels, indicating that the charge density, pore size or crosslinking density did not affect LYZ release mechanism. Under alkaline conditions n was ~ 1 , indicating Case II diffusion mechanism. Such faster protein release is caused mainly by the hydrogels hydrolysis.

3.4. Bactericidal activity of LYZ loaded hydrogels films

Lysozyme acts on bacterial cell wall, causing cellular lysis (Chipman & Sharon, 1969). The activity of LYZ loaded XNT or XCA hydrogels films, expressed as $\Delta\tau$, at pH 6.5 is presented in Fig. 5A, along with the activity of free LYZ. In the hydrogels LYZ molecules retain $\sim 60\%$ of the activity of free LYZ. For comparison, bare XNT or XCA hydrogels presented negligible activity. Particularly in the case of proteins delivery, it is important that the proteins do not undergo conformational changes upon loading or releasing process. Fig. 5B shows a typical CD spectrum obtained for LYZ loaded XNT hydrogel after subtraction of CD spectrum of XNT hydrogel swollen with water, where the characteristic negative bands at 209 nm and 215 nm appeared, indicating preservation of native conformation (Knubovets, Osterhout, Connolly, & Klibanov, 1999). The highly hydrated network offers a suitable environment to avoid denaturation (Pancera, Gliemann, Schimmel, & Petri, 2006).

The application of a wound dressing may be due to a large number of reasons, as, for instance, stopping blood flow and pain, enhancing healing process, protecting from infection, preventing

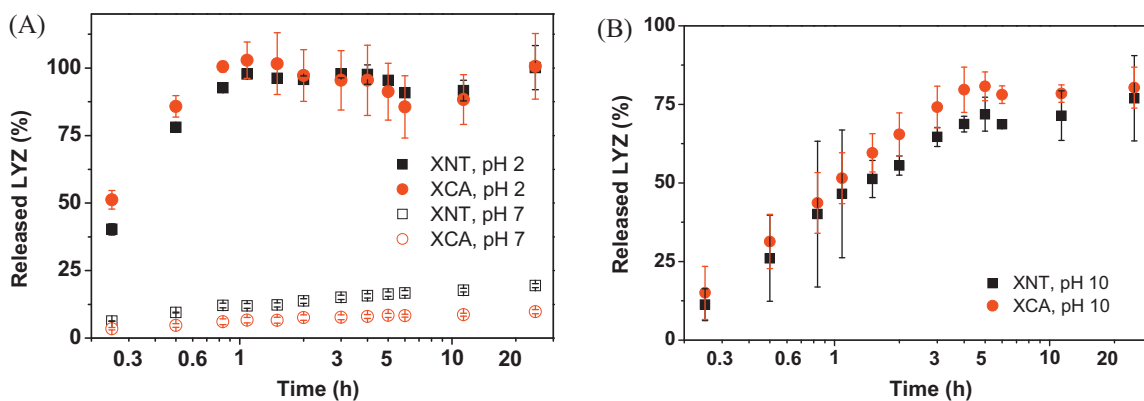


Fig. 4. Cumulative release of LYZ from XNT (black square) and XCA (red circle) hydrogels at (A) pH 2.0 (full symbols) and pH 7.0 (open symbols) and (B) pH 10.0 as a function of time. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

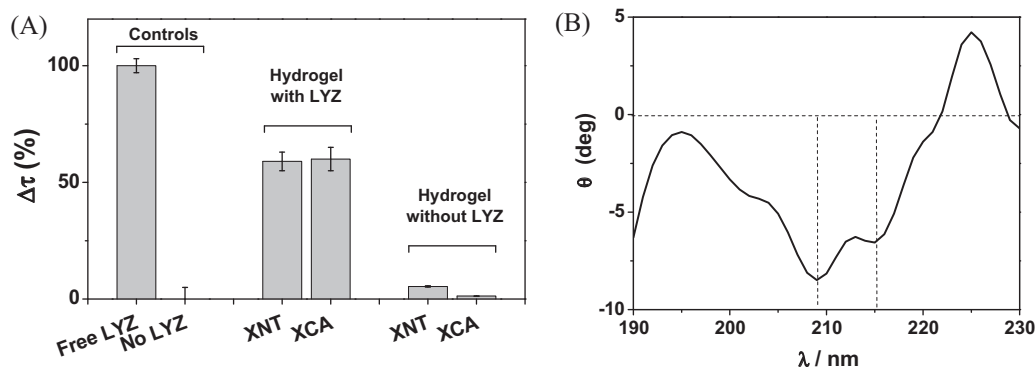


Fig. 5. (A) $\Delta\tau$ caused by bactericidal activity and (B) typical CD spectrum obtained for LYZ loaded XNT hydrogel after subtraction of CD spectrum of XNT hydrogel swollen with water.

adhesion of foreign particles and absorbing exudate. The ability to prevent infections is one of the most important utility of a dressing. Most wound infections are caused when bacteria reaches the wound attaching to the tissue, slowing the healing process and causing irritation. In addition, potential generalized infections which could enter the organism through the wound may be more serious than the wound itself.

The bactericidal activity observed for LYZ-loaded XNT and XCA hydrogels films (Fig. 5A) can be useful for applications as wound dressings, because as other hydrogel systems, they combine softness, humidity maintenance and porous structure, which allows gas exchange and speeding healing process. Moreover, XNT and XCA hydrogels films high adherence onto skin (Fig. 6) favors this application.

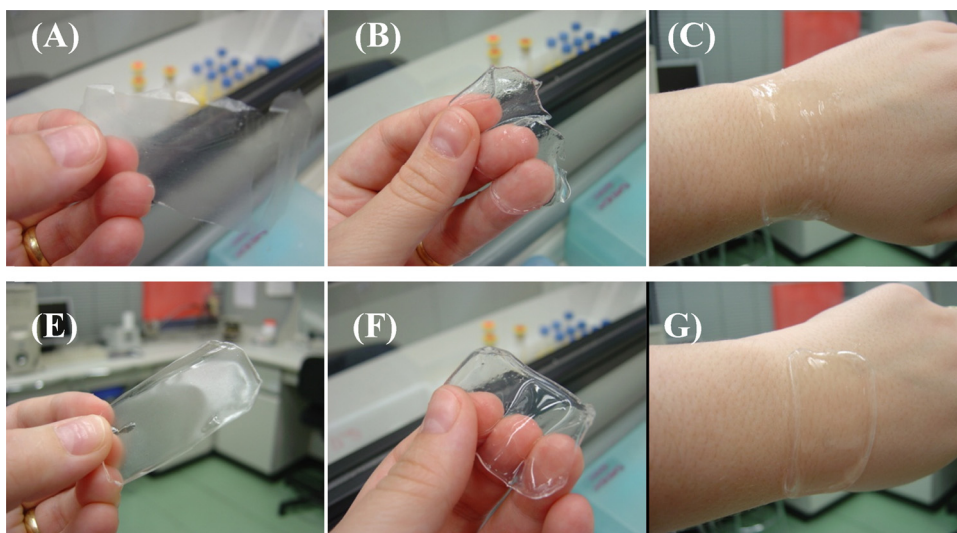


Fig. 6. Photographs of (A) dried XNT hydrogel, (B) swollen XNT hydrogel and (C) swollen XNT hydrogel adhered to skin; (D) dried XCA hydrogel, (E) swollen XCA hydrogel and (F) swollen XCA hydrogel adhered to skin.

4. Conclusions

The molecular conformations of xanthan chains in swollen hydrogels films were determined by CD measurements, evidencing substantial conformational differences between xanthan (XNT) and xanthan-citric acid (XCA) hydrogels. In XNT hydrogels xanthan chains keep ordered conformation (helices). In XCA hydrogels xanthan chains assume disordered conformation (coils), exposing a large number of negative charges. Upon increasing the ionic strength the ordered conformation of xanthan chains in XNT hydrogels was partially lost, while in the case of XCA hydrogels, a disorder order transition was observed, providing evidences that the charge density affects the conformational state of crosslinked xanthan chains. Thus, CD proved to be a powerful technique to determine not only the conformational state of macromolecules free in solution, but also of crosslinked swollen chains.

The ability of XNT and XCA hydrogels films to load and release proteins was controlled by hydrogels and proteins net charges. For proteins with low *pI* values, such as BSA, XNT hydrogels showed to be more adequate than XCA due to the lower charge density, which avoids electrostatic repulsion. The delivery of BSA from XNT was complete after 1 h, in the pH range from 2 to 10, except for pH 4.8, which is very close to its *pI*. For proteins with high *pI*, as for instance, LYZ, both hydrogels films are efficient as carriers. The LYZ release at pH 7.0 was very low due to electrostatic attraction between LYZ and hydrogels. The LYZ loaded hydrogels films presented substantial bactericidal activity, making them useful as bactericidal coatings or wound dressings. The preservation of LYZ native structure after hydrogels loading opens the possibility to load other enzymes in the hydrogels without activity loss.

Acknowledgements

We thank Brazilian Agencies CAPES Rede Nanobiotec, CNPq, FAPESP (process # 2010/13034-2 and # 2010/51219-5) for the 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.2013.10.039>.

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