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Synthesis and characterization of a novel chitosan-*N*-acetyl-homocysteine thiolactone polymer using MES buffer

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ABSTRACT

We report a new "green" approach to synthesize a novel thiolated chitosan conjugate, chitosan-*N*-acetyl-homocysteine thiolactone (chitosan-AcHcys) using a "Good's buffers", 2-(*N*-morpholino)ethanesulfonic acid (MES). After that, the crosslinked Xr–chitosan-AcHcys was obtained only in the presence of air, without other reactants. The chitosan-AcHcys spectrum shows a partial incorporation of the thiolactone onto the polymer backbone. The derivative thermogravimetric analysis confirmed that chitosan-AcHcys is slightly less stable than starting chitosan; however, the peak profile is broadened which is indicative of deeper changes in the thermal degradation process. Also, aqueous dispersions with different concentrations of the crosslinked material (Xr–chitosan-AcHcys) were prepared and rheologically characterized. All aqueous dispersions are viscoelastic fluid with shear-thinning behavior. The viscosity of the dispersions (1–7% of chitosan-AcHcys) increases as a function of polymer concentration. So, we have achieved to disperse a high concentration of thiolated-chitosan derivative in water with different rheological characteristics, which could affect the drug release.

1. Introduction

The biopolymer chitosan is obtained by alkaline deacetylation of chitin, which is one of the most abundant polysaccharides in nature (Felt, Buri, & Gurny, 1998). Its multidimensional and highly functionalized structure, as well as its unique properties makes it a biomaterial of wide ranging applications in biomedicine and other industrial areas (Chuang, Don, & Chiu, 2012; Hejazi & Amiji, 2003; Kean & Thanou, 2010; Pillai, Paul, & Sharma, 2009). Particularly, its widely use as a pharmaceutical excipient is due to its favorable properties, such as enzymatic biodegradability, non-toxicity, low-cost and biocompatibility (Wang, Li, Tao, Guo, & Yan, 2006).

From a chemical standpoint, the incorporation of new chemical groups or moieties to chitosan provides versatile materials with specific functionalities and modified physical and biological properties. Thus, a large number of chitosan derivatives have been prepared through modifications of the primary (C-6) and secondary (C-3) hydroxyl groups present on each repeating unit or the amine (C-2) group present on deacetylated units (Muzzarelli, 1977; Roberts, 1992). The active primary amine groups can be derivatized by the use of mild reaction conditions, as shown by early work on aldehydo-acids (typically glyoxylic acid, yielding N-carboxymethyl chitosan) and keto-acids (typically levulinic acid yielding 5-methyl pyrrolidinone chitosan) in the presence of NaBH4 at room temperature (Muzzarelli, Tanfani, Emanuelli, & Mariotti, 1982; Muzzarelli, Ilari, & Tomasetti, 1993). Alternative strategies aimed at increasing the solubility of chitosan can be exemplified by the introduction of various long hydrophilic segments into its structure such as poly(ethylene glycol) (PEG), and polyvinylpyrrolidone and dextran (Park et al., 2000, 2001, 2003; Yalpani & Hall, 1984). Those segments may hinder the polar groups from chitosan to get close enough to form hydrogen bonds.

Other chitosan derivatives are based on the immobilization of thiol-bearing moieties onto the polymeric backbone of chitosan (Baumann & Faust, 2001; Bernkop-Schnürch & Hopf, 2001; Bernkop-Schnürch & Krajicek, 1998; Bernkop-Schnürch, Brandt, & Clausen, 1999; Bernkop-Schnürch, Hornof, & Zoidl, 2003; Hornof, Kast, & Bernkop-Schnürch, 2003; Kast & Bernkop-Schnürch, 2001; Thanou, Nihot, Jansen, Verhoef, & Junginger, 2001). To date, five

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different thiolated chitosan derivatives have been synthesized: chitosan-thioglycolic acid conjugates (Bernkop-Schnürch, & Hopf, 2001; Hornof et al., 2003), chitosan-cysteine conjugates (Bernkop-Schnürch et al., 1999), chitosan-glutathione conjugates (Saremi, Atyabi, Akhlaghi, Ostad, & Dinarvand, 2011), chitosan-4-thiobutyl-amidine (chitosan-TBA) conjugates (Bernkop-Schnürch et al., 2003), and chitosan-homocysteine conjugates (Juntapram, Praphairaksit, Siraleartmukul, & Muangsin, 2012). Thus, the main process to incorporate of SH-bearing moieties into the chitosan structure has been conducted via the coupling reaction between the amine groups from chitosan and the carboxylic acid groups from —SH-bearing reagents at acid pH, where the use of a water-soluble carbodiimide was needed. This methodology presents several drawbacks: first, as the reagents have a thiol and carboxylic acid functional groups in their structures, a self-coupling reaction to generate a thioester bond is feasible. Secondly, during the coupling reaction a secondary byproduct is generated by transformation of the carbodiimide into its urea derivative. Moreover, the additional use of another reagent, the *N*-hydroxysuccinimide (NHS) is often necessary to improve its efficiency.

The other option explored to incorporate thiol groups into the polymer backbone is the nucleophilic attack of the amine groups to electrophilic reagents such as 2-iminothiolane (Traut's reagent). However, in all cases, a two-step solubilization process is required with the use of an acid to dissolve the polymer and subsequent partial neutralization with sodium hydroxide up to the required pH.

As far as we aware, there is just one attempt in the use of homocysteine thiolactone or any of its derivatives to prepare a new thiolated chitosan (Juntapram et al., 2012). The covalent binding of homocysteine thiolactone onto the amino groups of chitosan is aimed using imidazole to form a reactive intermediate. Despite the large amount of imidazole used (weight ratio chitosan–thiolactone–imidazole 1:1:0.68), non-significant incorporation of the thiolactone on the chitosan backbone was achieved. We believe that just as the free amine groups from chitosan can react with the thiolactone moiety, the same can occur with the free amine groups present in the unprotected homocysteine thiolactone, leading to competitive self-coupling reactions. This last fact should have had a marked impact in the final incorporation of thiol groups in the polysaccharide, and thus resulting in a low yield of this process. Therefore, we aim to use a reagent with the amine groups conveniently blocked (the *N*-acetyl-homocysteine thiolactone) to overcome these drawbacks and lead to an improvement in the degree of functionalization of chitosan.

"Green Chemistry" (GC) also called "Sustainable Chemistry" is the design of chemical products and processes that reduce or eliminate the use and generation of hazardous substances (Anastas, 1998).

Looking forward to a "green" approach for the preparation of thiolated chitosan, the solubilization of the macromolecule via a non-toxic system (avoiding the use of acids) is required. In that sense, most biological buffers widely used in cell culture and other biological applications nowadays were developed by Good and coworkers (Ferguson et al., 1980; Good & Izawa, 1972). They described a series of zwitterionic buffers, also called "Good's buffers", which are non-toxic to cells and present pK_a values close to physiological pH. Among them, 2-(*N*-morpholino)ethanesulfonic acid (MES) is a bulky molecule used as a buffering agent in biology and biochemistry with a pK_a value of 6.15 at 20 °C.

To carry out modifications in chitosan structures where the amine group acts as a nucleophile, there are two opposing aspects to be taken into account: first, the (partial or total) solubilization of the polymer is needed and, if not all, at least part of the amine groups have to be protonated; secondly, the reactivity of chitosan as nucleophile is based on the presence of unprotonated free amine groups. Therefore, such pH is required in which both requirements are convened. Applying the Henderson–Hasselbalch equation, MES could be an excellent candidate to prepare the buffer chitosan solutions at pH 6.0.

Thus, the aim of this work is to report a new "green" approach for the preparation of chitosan-*N*-acetyl-homocysteine thiolactone conjugate (chitosan-AcHcys) in the presence of a "Good's buffer", 2-(*N*-morpholino)ethanesulfonic acid (MES), and its crosslinked Xr–chitosan-AcHcys in the presence of air. The use of other reactants, reagents or catalysts is prevented. The physico-chemical characteristics of the conjugated obtained will be evaluated. Also, aqueous dispersions with different concentrations of the crosslinked material (Xr–chitosan-AcHcys) will be rheologically characterized.

2. Materials and methods

2.1. Materials

All chemicals used were from Aldrich Chemical Co. Solvents were dried and purified when necessary, by appropriate standard procedures. Chitosan with a deacetylation degree of 75% and molecular weight ranging from 190 kDa to 375 kDa, (based on viscosity values) was used as received. *N*-Acetyl-D,L-homocysteine thiolactone was handled under inert atmosphere.

2.2. Methods

2.2.1. Modification of chitosan with N-acetyl-D,L-homocysteine thiolactone. Preparation of chitosan-AcHcys conjugate

In a typical procedure, chitosan with a deacetylation degree of 75% (chitosan, 1.5 g, 6.55 mmol of free amine groups) was charged in a round-bottom flask provided with an inlet of Ar; an aqueous buffer solution of 2-(*N*-morpholino)ethanesulfonic acid (MES, 150 mL, 0.05 M, pH 6.0) was added and the mixture was stirred to homogenization at 25 °C to a final concentration of 1.0% (w/v). The solution was then deoxygenated by bubbling it with argon for 60 min. Next, *N*-acetyl-D,L-homocysteine thiolactone (AcHcysTL, 835 mg, 5.24 mmol, molar ratio related to free amine groups 0.8) was added and the reaction proceeded for 48 h in dark, under inert atmosphere at 25 °C. Three different batches of these conjugates were synthesized for comparative purposes. To determine the degree of modification, the residue was purified by dialysis against deionized water and dried by lyophilization, giving the title compound as a white solid. The freeze-drying process for all the samples was carried out under the same conditions using a VirTis lyophilizer 4KBTEL. Samples (50 mL of a 1%, w/v aqueous solution) were previously frozen under nitrogen in a one-liter round-bottom flask. The temperature and the pressure stabilized at −100 °C and 100 mTorr, respectively for 30 min and then the samples were liophilized for 48 h.

2.2.2. Preparation of crosslinked chitosan-AcHcys conjugate (Xr–chitosan-AcHcys)

In a typical procedure, the aqueous buffer solution at pH 6.0 of the above mentioned thiolated chitosan was oxygenated by bubbling air into the solution at 25 °C for 48 h. Next, the residue was dried by lyophilization, giving a white solid that was held in reserve for further preparation of aqueous dispersions at different concentrations of the powder obtained.

2.2.3. Preparation of aqueous dispersions of Xr–chitosan-AcHcys

The dispersions were prepared at pH 5.0 to achieve the desired concentration of Xr–chitosan-AcHcys (1%–7%, w/v). The general procedure is detailed next: the calculated amount of Xr–chitosan-AcHcys was put into a graduated flask, 8 mL of double distilled water were added and the mixture was gently stirred. The pH of the

media was adjusted at pH 5.0 by the addition of a very dilute aqueous acidic solution (HCl 0.05 M). Then, distilled water was added up to a final volume of 10 mL. The mixture was shaken for 4 h at room temperature and then incubated overnight at 37 °C to remove entrapped air bubbles.

2.2.4. Characterization of chitosan-AcHcys conjugate

2.2.4.1. Fourier transformed infrared spectroscopy (FT-IR). To investigate the chemical reaction between chitosan and AcHcysTL in an aqueous buffer solution of MES, IR spectra of chitosan, AcHcysTL, MES, and chitosan-AcHcys conjugate were recorded on a Jasco FT/IR 4200 spectrometer equipped with ATR in the range between 4000 and 600 cm^{-1}.

2.2.4.2. Thermogravimetric analysis (TGA). The thermal stability of each of the samples was evaluated using thermogravimetric analyses (TGA). These experiments were performed under a nitrogen atmosphere (flow rate 100 mL min^{-1}) with a TA Instruments SDT Q600 at a heating rate of 10 °C min^{-1} from 25 to 700 °C.

2.2.4.3. Differential scanning calorimetry (DSC). The thermal behavior of the polymers was examined by differential scanning calorimetry (DSC), using a TA Instruments DSC Q200 calibrated with indium. Aluminum hermetic pans were used throughout the study. After loading the samples, the pans were sealed with a dry weld. DSC data were obtained from samples of 4–6 mg at heating/cooling rates of 10 °C min^{-1} under a nitrogen flow (50 mL min^{-1}). The experimental method started with an initial 20-min isothermal period at 25 °C to allow equilibration of the sample to the programmed temperature, then heating to 180 °C. Temperature modulated differential scanning calorimetry (TMDSC) was used to determine the glass transition temperatures (T_g) of chitosan and the new synthesized thio-conjugates. The following parameters were selected: modulation amplitude of 0.5 °C and a 60 s modulation period with a 2 °C min^{-1} underlying heating rate. The experimental method consisted of an initial 20-min isothermal period at 25 °C to allow equilibration of the sample to the programmed temperature modulation, then heating to 130 °C.

2.2.4.4. Determination of sulfur content in the chitosan-AcHcys conjugates by elemental analyses. The degree of modification was determined by Elemental Analyses in the Microanalyses Laboratories of the CITIUS Service (University of Seville, Spain). All the measurements were performed in triplicate in each experiment. The total amount of sulfur in the thiolated conjugates was determined by elemental analysis using an Analyzer LECO CHNS 932 and a Sartorious Micro Balance, M2P. Samples of 10 mg were burned at 1000 °C in an oxygen flux and the generated SO_2 detected by infrared measurement. The amount of water encountered in the conjugates was previously determined by TGA, and then, taken into account for the calculations of the total sulfur content in the referred conjugates from the elemental analysis data obtained.

2.2.5. Rheological characterization of Xr–chitosan-AcHcys aqueous dispersions

The evaluation of viscoelastic properties was made in aqueous dispersions using six different concentrations (1%–7%) of the new solid Xr–chitosan-AcHcys. For this purpose, multi-step flow curve measurements were run using a controlled stress rheometer (AR-2000, TA Instruments, New Castle, USA), using a geometry of 60 mm diameter with smooth surface. The temperature was maintained constant with the Peltier plate (25 ± 0.1 °C). The experimental protocol began with the determination of the linear viscoelastic region of systems by oscillatory stress sweeps at 1 Hz of frequency. After that, one oscillatory stress was selected to allow work in non destructive conditions. A frequency sweep on range from 0.01 rad/s to 628.3 rad/s was carried out. The analysis of results is based on the storage (G') and loss (G'') moduli, which are related to the elastic and viscous components, respectively. On the other hand, flow and viscosity curves were measured by rotational shear assay.

According to the shear stress/shear rate behavior, a rheological classification of materials can be made. Among them, Newtonian (Deem, 1998) and non-Newtonian (Lucero, Leon, Vigo, & Rabasco, 1991) materials are the most important in the pharmaceutical field.

Different theoretical models can be used to treat the rheological results. Cross flow model (Lucero, Claro, Casas, & Jiménez-Castellanos, 2013) has been used to describe the shear-thinning or pseudoplastic behavior by Eq. (1):

$$\eta(\dot{\gamma}) = \frac{\eta_0}{1 + (\lambda\dot{\gamma})^m}$$

where η_0 is the zero-shear rate viscosity (Pa s), λ is a structural relaxation time or time constant(s) and m is a shear index dimensionless which indicated the degree of shear thinning. When m approaches zero the liquid is Newtonian behavior, while the most shear-thinning liquids have a value of m approaching unity (Álvarez-Manceñido et al., 2006; Rudraraju & Wyandt, 2005).

3. Results and discussion

3.1. Preparation of chitosan-AcHcys conjugate and its crosslinked material Xr–chitosan-AcHcys

In this work, AcHcysTL has been used for the incorporation of thiol moieties into the polymer backbone of chitosan (Fig. 1) (de Paz, Jiménez-Castellanos, Lucero, Casas, & Ferris, 2013). The reaction was carried out following the method recorded above in which a 1% (w/v) of chitosan was prepared in an aqueous MES buffer solution at 25 °C. Three batches of the new polymer (chitosan-AcHcys) were synthesized to confirm the reproducibility of this method.

As far as we aware, this is the first synthetic procedure for the preparation of thiolated chitosan derivatives carried out in full with tolerant biological molecules. The need of an acid media to dissolve the starting chitosan and later pH adjustment by the addition of sodium hydroxide was circumvented. Either, high temperatures were not required in any step of the synthesis avoiding the possible spoil of the starting material. This one pot reaction does not require intermediate purification.

MES was the buffer of choice because of its pK_a and its chemical structure which avoid the reorganization of the hydrogen bonds in extensive chitosan segments. A suspension of chitosan in MES buffer (pH 6.0) was stirred for 1 h. At this pH nearly 50% of the amino groups are uncharged and available to carry out the nucleophilic attack over the AcHcysTL. The reaction was stirred for 48 h and the incorporation of free thiol groups in the new conjugate (chitosan-AcHcys) was achieved (Fig. 1). The disulfide linkages were chemically formed between the —SH moieties when an airflow bubbled through the suspension to give the crosslinked material (Xr–chitosan-AcHcys). The final solution was lyophilized and the resulting material was stored at 25 °C. In summary, only airflow was necessary to prepare the crosslinked material from the chitosan-based conjugate.

3.2. Characterization of chitosan-AcHcys conjugate

3.2.1. Fourier transformed infrared spectroscopy (FT-IR)

Fig. 2 shows the FTIR spectra of the unmodified chitosan, *N*-acetyl-D,L-homocysteine thiolactone and the chitosan-AcHcys conjugate. In the IR spectrum of chitosan, hydrogen bonded hydroxyl groups provide a broad strong band centered at 3356 cm^{-1}, which is overlapped with the stretching bands corresponding to the N—H bonds from amine (—NH_2) and amide groups.

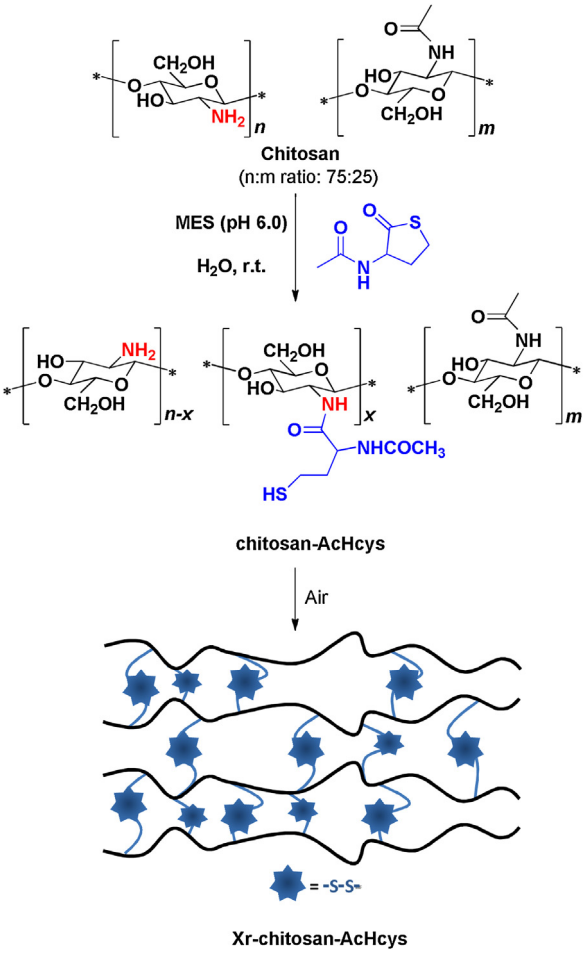

Fig. 1. Reaction scheme for the synthesis of chitosan-*N*-acetyl-homocysteine thiolactone conjugate and its cross-linking in the presence of air.

Peak width is due to the presence of water in the sample and subsequent hydrogen bonding. The amide I band resultant from st C=O vibrations is found at 1653 cm^{-1}. The band at 1593 cm^{-1} is attributed to the absorption of st N—C=O (amide II) as well as the δ N—H vibrations due to the amine and amide groups. Other important absorption band at 1062 cm^{-1} can also be observed (st C—O).

The *N*-acetylhomocysteine thiolactone spectrum showed a stretching narrow band corresponding to the N—H amide groups at 3273 cm^{-1} and an absorption band due to the C=O group at 1698 cm^{-1}. Those peaks can be found in the raw materials obtained after the coupling reaction of chitosan with the thiolactone, and consequently, it can be deduced that a partial incorporation of the thiolactone onto the polymer backbone took place. Hence, after the grafting reaction of the thiolactone onto the C2-amine groups of chitosan, the polymer was dialyzed, and the previously mentioned bands from thiolactone were not found as high as the dialyzed conjugate bands; however, the characteristic signal at 1652 cm^{-1}, attributed to the stretching vibration of the C=O amide (amide I band) was stronger than those seen in chitosan, which can be attributed to the additional amide group formed. The band at 1555 (NH δ, amide) was also present in the IR spectrum of the dialyzed product. The absorption band due to S—H stretching vibrations of free thiol groups was not observed since the oxygenation of the sample was carried out before the IR measurement and the previous disulfide bonds formation was achieved. These results were consistent with the successful preparation of partially thiolated chitosan.

3.2.2. *Thermal studies: thermogravimetric analysis (TGA) and differential scanning calorimetry (DSC)*

Both thermogravimetric analysis (TGA) and differential scanning calorimetry (DSC) studies were carried out on chitosan, *N*-acetyl-D,L-homocysteine and chitosan-AcHcys conjugate.

TG curves of the chitosan and *N*-acetyl-D,L-homocysteine thiolactone show a one-step degradation scheme at 308 °C and 250 °C respectively, characterized by narrow and well-defined peaks. As a result of the coupling reaction, the new synthesized chitosan-AcHcys conjugate shows a maximum degradation temperature at 304 °C. Although this value is slightly shifted to lower temperatures from the starting material, the peak profile is broadened which is indicative of deeper changes in the thermal degradation process. Fig. 3 represents the derivative thermogravimetric analysis (DTG) curves of chitosan, *N*-acetyl-D,L-homocysteine thiolactone and the new synthesized chitosan-AcHcys conjugate. This figure provides a better visualization of the phenomena mentioned above. Thus, the decomposition onset temperatures with a 10% weight loss (T_d^0) of chitosan and its conjugate differ in 26 °C (293 °C and 267 °C, respectively) which confirmed that the new conjugate is slightly

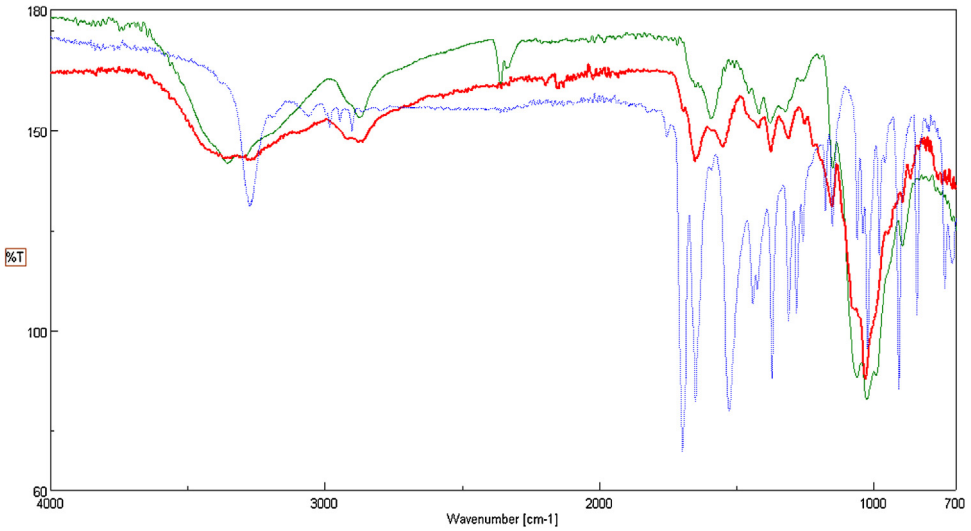

Fig. 2. Fourier transform infrared spectroscopy spectra of chitosan, *N*-acetyl-D,L-homocysteine thiolactone and chitosan-*N*-acetyl-homocysteine thiolactone conjugate.

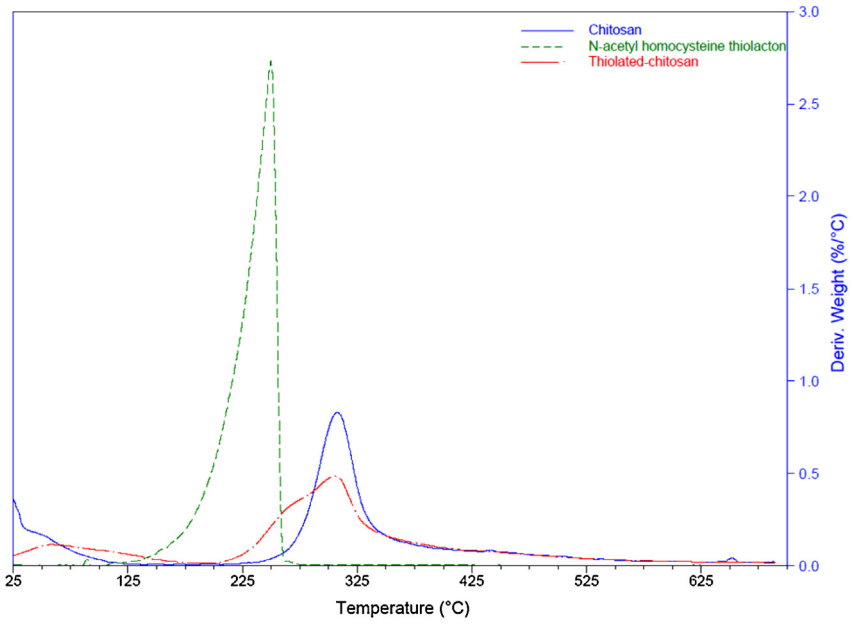

Fig. 3. Derivative thermogravimetric analysis (DTG) curves of chitosan, *N*-acetyl-D,L-homocysteine thiolactone and the new synthesized chitosan-AcHcys conjugate.

less stable to thermal degradation than the starting chitosan used. It can also be inferred from those results that the incorporation of the thiolactone into the chitosan chains took place successfully. Additionally, the water content of the chitosan-AcHcys conjugate was determined by TGA. The first degradation stage at about 60 °C was due to the water loss and presented an associated weight loss from 12.5% to 13.5% depending on the analyzed batch. These values were taken into account for further studies related to the total sulfur content of the new materials from elemental analyses.

T_g of conjugate (38 °C) was slightly lower than the chitosan (40 °C). Ratto et al. (1995) demonstrated that the T_g values of chitosan depend on its water content. According to this theory, the observed decrease in T_g in the modified chitosan-AcHcys could be related not only to the more bulky side chains incorporated but also to the higher water incorporation encountered in the new compounds.

3.2.3. Determination of sulfur content in the chitosan-AcHcys conjugate by elemental analyses

One of the features to study in the newly synthesized thiolated-polymers is the degree of functionalization achieved by reaction with AcHcys thiolactone. The total sulfur content in the thiolated-chitosan conjugate was determined by elemental microanalyses. The three batches of thiolated chitosan (chitosan-AcHcys) used in the present work were analyzed by Elemental Microanalysis and the data are recorded in Table 1.

Theoretical elemental analysis of raw chitosan and chitosan-AcHcys were calculated. Water content, found according to the TGA studies for both, the original and modified chitosan, was incorporated to those analyses to compare theoretical and experimental data. A good agreement between the estimated and the found analysis was observed in every sample.

The water content encountered in the commercial chitosan and the three batches of chitosan-AcHcys conjugates were estimated by TGA. The measurements of volatiles were carried out in triplicate and the water content encountered for the commercial chitosan was 10.9 ± 0.1%, whereas this rate increased for the chitosan-AcHcys conjugates (from 12.5% to 13.4%). The values were consistent with those inferred from the elemental analyses with an underestimation of the data based on TGA of 1%–2% in water content. It is noticeable that, although the new conjugate has some less-hydrophilic side chains than the starting chitosan, the total water content is increased, probably because the chains are looser than the starting chitosan enabling better water penetration into the polymer structure.

Coupling reactions performed between chitosan and the thiolactone showed that the total amount of immobilized sulfur content onto the final polymer is constant in the assays tested. As shown in Table 1, the sulfur containing moieties immobilized on the polymer ranged from 550 μmol/g to 557 μmol/g.

3.2.4. Rheological characterization

Different amounts of Xr–chitosan-AcHcys were used to obtain the dispersions in which only water is used as a unique vehicle (1–7%, w/v). These aqueous dispersions were stable at least during 7 days.

Fig. 4 shows oscillatory stress sweeps of aqueous dispersions at different concentrations of Xr–chitosan-AcHcys (1–7%), in order

Table 1
Elemental microanalysis data, percentages of moisture determined by TGA and elemental microanalysis (E. A.), and immobilized total sulfur content values from commercial chitosan and the three batches of thiolated chitosan (chitosan-AcHcys) synthesized.

Batches	Theorical analysis				Experimental analysis				Water content (%)		Mass of S/mass of polymer (mg/g)	Immobilized total S content (μmol/g)
	%C	%H	%N	%S	%C	%H	%N	%S	TGA	E. A.		
Commercial chitosan	40.02	7.29	7.18	–	39.83	6.97	7.22	–	10.9 ± 0.1	12	–	–
Chitosan-AcHcys (a)	39.06	7.30	7.06	1.55	38.88	6.77	7.05	1.53	12.5 ± 0.1	14	17.8	557
Chitosan-AcHcys (b)	39.08	7.30	7.06	1.47	38.88	7.02	7.11	1.51	12.8 ± 0.1	14	17.6	550
Chitosan-AcHcys (c)	38.44	7.36	6.95	1.45	38.61	7.02	7.03	1.50	13.4 ± 0.2	15	17.7	554

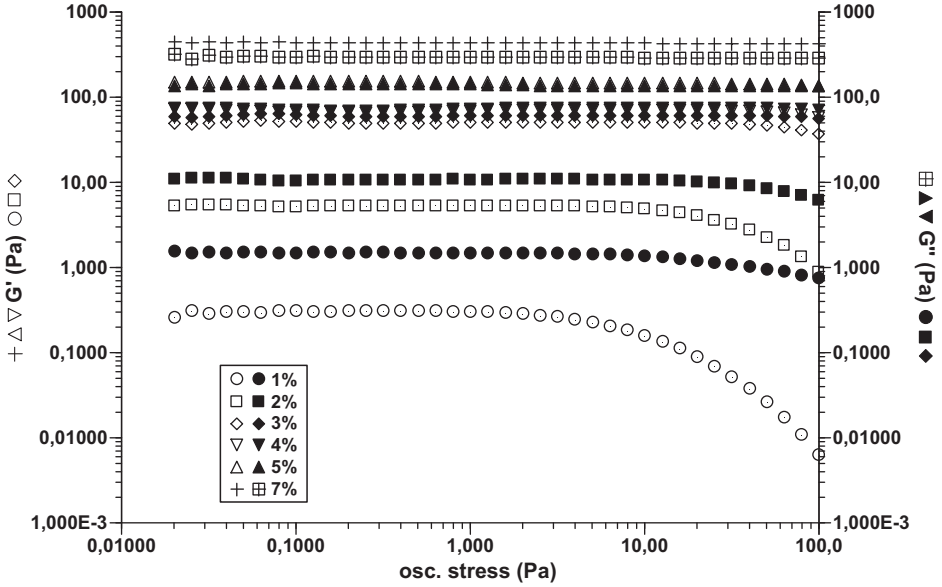

Fig. 4. Storage (G') and loss (G'') moduli values of dispersions at different concentrations of crosslinked chitosan-*N*-acetyl-homocysteine thiolactone.

to determine the linear viscoelastic region, where elastic (G') and viscous (G'') components have constant values and are independent of the applied oscillatory stress (Ferrari et al., 2001). So, systems with 3%–7% Xr–chitosan-AcHcys have a linear viscoelastic region throughout the stress range applied (0.01–100 Pa), while 1% and 2% of crosslinked polymer dispersions display a shorter region, only until 1 or 10 Pa, respectively. Moreover, there are an increase of the G' and G'' values with the Xr–chitosan-AcHcys concentration increment.

Frequency sweeps of storage (G') and loss (G'') moduli vs. frequency sweep of aqueous dispersions at different concentrations of Xr–chitosan-AcHcys are shown in Fig. 5. In all samples, G' and G'' increase with the applied frequency over the range tested. Therefore, all these samples are viscoelastic fluids, but with different predominance depending on the crosslinked polymer concentration. On the other hand, the dispersions with 3% and 4% of Xr–chitosan-AcHcys have two crossover points of the curves (known as the reticulation point) where a predominance of the elastic component (G') begins, at frequency values of 0.024 and 27.21 rad/s for 3%; and 0.097 and 11.46 rad/s for 4%. There is a viscous predominance (G'') for the derivatives with lower concentration of Xr–chitosan-AcHcys (1%–4%); however, from 5% is a trend toward the predominance of the elastic component, being more evident in 7%.

By representing the viscosity (Pa s) versus shear rate (1/s) information is obtained about the flow behavior of the Xr–chitosan-AcHcys (Fig. 6). It has been observed that all preparations have a non-Newtonian behavior with shear-thinning flow (pseudoplastic flow), in which the viscosity decreases with the increased shear rate. The systems become more fluid. Moreover, there is a significant increase in the viscosity curves of the dispersions as a function of crosslinked polymer concentration, varying from 0.1 to 1.10^5 Pa s.

Applying rheological theoretical models, the Cross equation has been the best fit for the dispersions (Lucero et al., 2013), whose principal parameters are shown in Table 2. The viscosity (η_0) increases when increases the Xr–chitosan-AcHcys concentration, in agreement with authors who worked with other polymers (Rudraraju

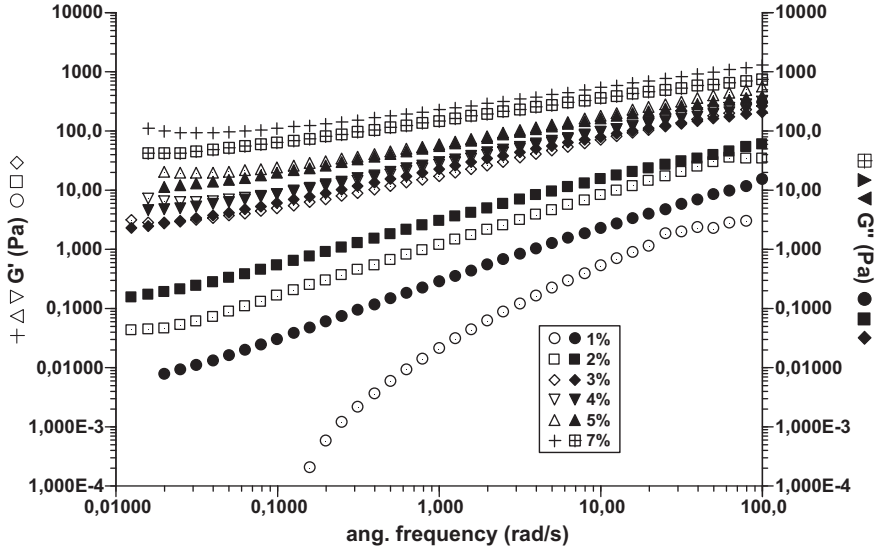

Fig. 5. Frequency sweeps of storage (G') and loss (G'') moduli vs. frequency sweep of dispersions at different concentrations of crosslinked chitosan-*N*-acetyl-homocysteine thiolactone.

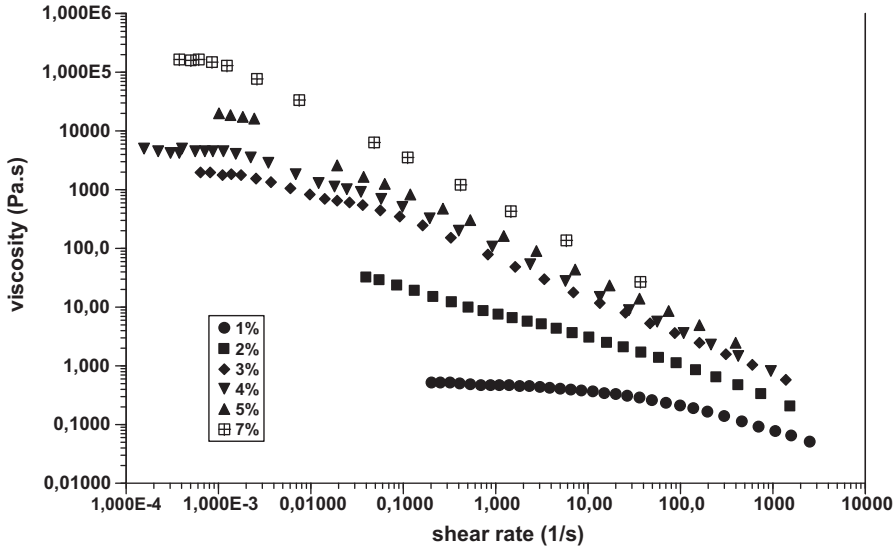

Fig. 6. Flow curves of dispersions at different concentrations of crosslinked chitosan-*N*-acetyl-homocysteine thiolactone.

Table 2
Parameters obtained from cross model: zero-shear rate viscosity (η_0), infinite time viscosity (η_∞), structural relaxation time (λ), shear index (m) and standard error (SE).

Concentration (%)	η_0 (Pa s)	η_∞ (Pa s)	λ (s)	m	SE
1	0.51	1.70×10^{-3}	0.022	0.569	5.57
2	210.4	5.73×10^{-7}	1217	0.454	13.72
3	2472.0	0.04	202	0.669	8.85
4	5677.0	0.08	306	0.708	10.37
5	3.51×10^{5}	8.15×10^{-5}	40,520	0.713	10.39
7	3.01×10^{5}	1.90	1462	0.860	9.58

& Wyandt, 2005; Sittikijyothin, Torres, & Gonçalves, 2005). The η_0 and m values increase with crosslinked polymer concentration increase in the aqueous dispersions. Moreover, m values are in all cases lower than 1, which indicates a shear-thinning behavior.

The relaxation time is related to the viscosity curve. The variations in this parameter could be justified by the big difference between the profiles for different polymer concentrations. It may be noted, the high relaxation time at 5%, which loses ten decades in viscosity values between η_0 and η_∞.

4. Conclusions

The preparation, via "green chemistry" (i.e. using natural resources), of a new thiolated chitosan-conjugate (chitosan-AcHcys) was achieved using MES buffer because of its suitable properties. FT-IR studies and thermal analysis of the new chitosan-AcHcys conjugate confirmed the incorporation of the thiol groups. Elemental Analysis also proved high sulfur content in the synthesized materials. Although the new conjugate has some less-hydrophilic side chains than the starting chitosan, the total water content increased, probably because the chains are looser than the starting chitosan, enabling better water penetration into the polymer structure. To obtain the crosslinked material Xr–chitosan-AcHcys, it was only necessary bubbling air over the thiolated-based chitosan conjugate, forming disulfide linkages between the —SH moieties. The aqueous dispersions obtained at different concentration of Xr–chitosan-AcHcys were stable at least for a week. The rheological studies show that the increase of crosslinked polymer concentration leads to a sequential change in the internal structure of the aqueous dispersions obtained, affecting both the flow properties and viscosity. These studies suggest the influence of polymer concentration on the drug release.

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