Preparation and Characterization of β-Cyclodextrin-Linked Chitosan Microparticles

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ABSTRACT: Hydrophobically modified chitosan containing β-cyclodextrin (CD) units was synthesized by using tosylated β-CD. The final product was characterized by Fourier transform infrared (FTIR) spectroscopy, elemental analysis and TGA, and rheometry. The polymer bearing β-CD moieties was used to obtain crosslinked microparticles by spray-drying which could then be used in a controlled release system for drugs. FTIR confirmed the formation of an amide linkage between cyclodextrin and chitosan. As fluorescence spectroscopy demonstrated, hydrophobic microenvironments were formed by chitosan bearing cyclodextrin in solution at lower concentrations than for chitosan. Rheometry and FTIR showed the crosslinking of the new polymer using genipin, a molecule of natural origin. Microspheres (MS) obtained by spray-drying showed narrow size distribution when β-CD was grafted onto chitosan and ξ-potential of MS was slightly lower although it remained positive. In conclusion, β-CD linked chitosan polymer can be considered as a very promising controlled drug delivery system for drugs. © 2011 Wiley Periodicals, Inc. J Appl Polym Sci 123: 3595–3604, 2012

Key words: chitosan modification; β-cyclodextrin; microencapsulation; biopolymer; drug delivery system

INTRODUCTION

Biodegradable microspheres (MS) based on chitosan are well known delivery systems for a wide range of drugs and peptides.[1,2] Among all the potentially useful polymers for this kind of drug delivery systems, polysaccharides as chitosan are a very attractive alternative mainly due to their low cost, biodegradability, and biocompatibility. Encapsulation of drugs in polymeric drug delivery devices based on chitosan serves as an effective tool not only to deliver drugs to an appropriate target, like particles produced using other polymers,[3] but also to increase their therapeutic efficacy.[4]

Chitosan is a naturally occurring polysaccharide [α(1→4) 2-amino-2-deoxy-β-D-glucan] obtained by the alkaline deacetylation of chitin. This copolymer of *N*-acetylglucosamine and glucosamine with a high degree of deacetylation (DDA) has favorable biological properties such as biodegradability, low cost and biocompatibility. Moreover, chitosan has primary amino groups at C-2 position of each monomeric unit, which allow the chemical modifications of chitosan. These highly reactive sites enable the grafting of molecules to provide a new or specific functionality. Many other modifications of chitosan have been made to improve its interesting properties in drug delivery,[5–8] although no further investigation has been carried out on the possibility of obtaining microparticles with potential release characteristics for drug delivery.

β-cyclodextrin (CD) is a cyclic oligosaccharide built of 7 D-glucose units. The D-glucose units are attached by α(1→4) bonds to form torus-like structures. Their cavities, which are of a hydrophobic nature, provide an ideal binding for drugs of adequate size, such as aromatic small organic molecules. Grafting cyclodextrin molecules into chitosan-reactive sites leads us to build a carrier which combines the effects of inclusion, size specificity, and transport properties of cyclodextrin as well as the controlled release ability of chitosan.

Initial burst effect is common when chitosan-based delivery systems are loaded with water soluble drugs and therefore their application for the controlled delivery of drugs in gastrointestinal tract is limited. Thus, to sustain the release of the bioactive substance, there is a need to crosslink the chitosan matrix due to its hydrophilic properties. Genipin was used in a one-step procedure for the preparation of crosslinked chitosan and β-CD-chitosan by

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spray-drying. Genipin is an aglucone of geniposide which is isolated from gardenia fruits (*Gardenia jasminoides ELLIS*). In *in vivo* studies, genipin has been shown to have a superior biocompatibility and slower degradation rate on chitosan implants than glutaraldehyde crosslinked MS.[9]

To date, various approaches have been proposed to link cyclodextrin to chitosan using different methods of preparation. In the work by Prabaharan and Mano, tosylated β-CD has been used to link chito.0-san at the 2-position of CD.[10] Previously, Chen and Wang[11] used the inclusion complex of β-CD-chitosan with iodine to release iodine slowly in rats. Other applications reported have been the immobilization of aromatic molecules in films made of this material to be used as chemical sensors.[12] In this study, an attempt has been made to introduce hydrophobic nature by modifying chitosan with CD.

The purpose of this work was to obtain chitosan microparticles bearing cyclodextrin cavities on their structure to (i) provide chitosan with a hydrophobic region for better inclusion of hydrophobic or low solubility substances, (ii) to decrease the highly cationic charge associated with chitosan, and therefore (iii) to produce particles as previously mentioned while maintaining the usual properties associated with chitosan such as adhesion and permeability through mucosa.[13,14]

In this article, the synthesis of β-CD-chitosan using tosylate intermediate is described. The material obtained was characterized by Fourier transform infrared (FTIR) spectroscopy, elemental analysis, and rheometry. Microparticles based on this polymer were obtained by one-step spray-drying and were crosslinked by genipin, a nontoxic and natural compound.

MATERIALS AND METHODS

Materials

Chitosan from Sinochem Qingdao Co., Qingdao, China was used for the preparation of CD-chitosan. *N*, *N*-dimethyl formamide, dichloromethane, sodium hydroxide, and ammonium chloride were purchased from Panreac, Spain. P-toluenesulphonic chloride and pyrene were supplied by Sigma-Aldrich (Spain). *p*-Toluensulphonic acid monohydrate was purchased from Avocado Research Chemicals, Lancashire, England. CD was supplied from Roquette, Spain. Silicagel MN Kieselgel 60*M* was delivered from VWR International (Barcelona, Spain). All other chemicals were analytical pure grade and used as delivered.

Methods

Synthesis of chitosan derivative bearing CD units

Preparation of CD tosylate. The reaction scheme for the synthesis of CD units grafted onto chitosan is shown in Figure 1. The cyclodextrin tosylate was prepared by using a modified method of Zhang et al.[15] as follows:

Ts_2O *p*-toluensulfonic anhydride: A mixture of tosyl chloride (80 g 0.43 mol) and tosic acid (20 g 0.11 mol) in 500 mL of dichloromethane was stirred overnight. The reaction mixture was filtered through silica gel. Pure *p*-toluensulfonic anhydride (Ts_2O) was obtained when the filtrate was precipitated from hexane.

Cyclodextrin 6-*O*-tosylate: 11.5 g of CD (10 m*M*) were dissolved and 4.9 g (15 m*M*) of *p*-toluensulfonic anhydride were added to the aqueous solution of cyclodextrin (250 mL) at room temperature for 2 h. A solution of NaOH (5 g, 50 mL water) was added. After 10 min, the unreacted Ts_2O was removed by filtration through a sintered glass funnel. The filtrate was brought to pH $\sim$ 8 by the addition of NH_4Cl and HCl 37%. Finally, the product was filtered and dried in a vacuum oven at 50°C.

β-cyclodextrin grafting onto chitosan.. Three grams of chitosan powder were weighed. The powder was added to dimethylformamide (DMF; 150 mL) and the mixture was stirred for 90 min. After that, 5 g of cyclodextrin tosylate was added and the mix was stirred for 72 h at 60°C. The product was concentrated partially in a vacuum. Then, the product was filtered in a glass sintered funnel #5. The solid was washed with water (150 mL) and dried under vacuum at 60°C using phosphorus pentoxide.

Characterization of β-CD-cyclodextrin-linked chitosan

The final product was characterized by elemental analysis, FTIR, rheometry, fluorimetry, and differential thermal analysis. 2% (w/v) of chitosan and 2% (w/v) of β-CD-chitosan were N-acetylated using acetic anhydride (3% w/v) in an aqueous AcOH-MetOH solution. After alkali treatment, the products were washed with water and desiccated to obtain fully acetylated chitosan and β-CD-chitosan.

Elemental analysis.

The elemental analyses of chitosan and β-CD-chitosan derivative were carried out using a Carlo-Erba 1108 Elemental Analyzer (Fisons Instruments, UK).

FTIR

IR spectra were recorded over the range 400–4000 cm^{-1} in a Bruker IFS 66V FTIR spectrometer (Germany) using samples which were mixed with KBr and then pressed to disk.

The DDA was determined by IR spectroscopic methods using a calibration line obtained by Shigemasa et al.[16] The values of different ratios of probe

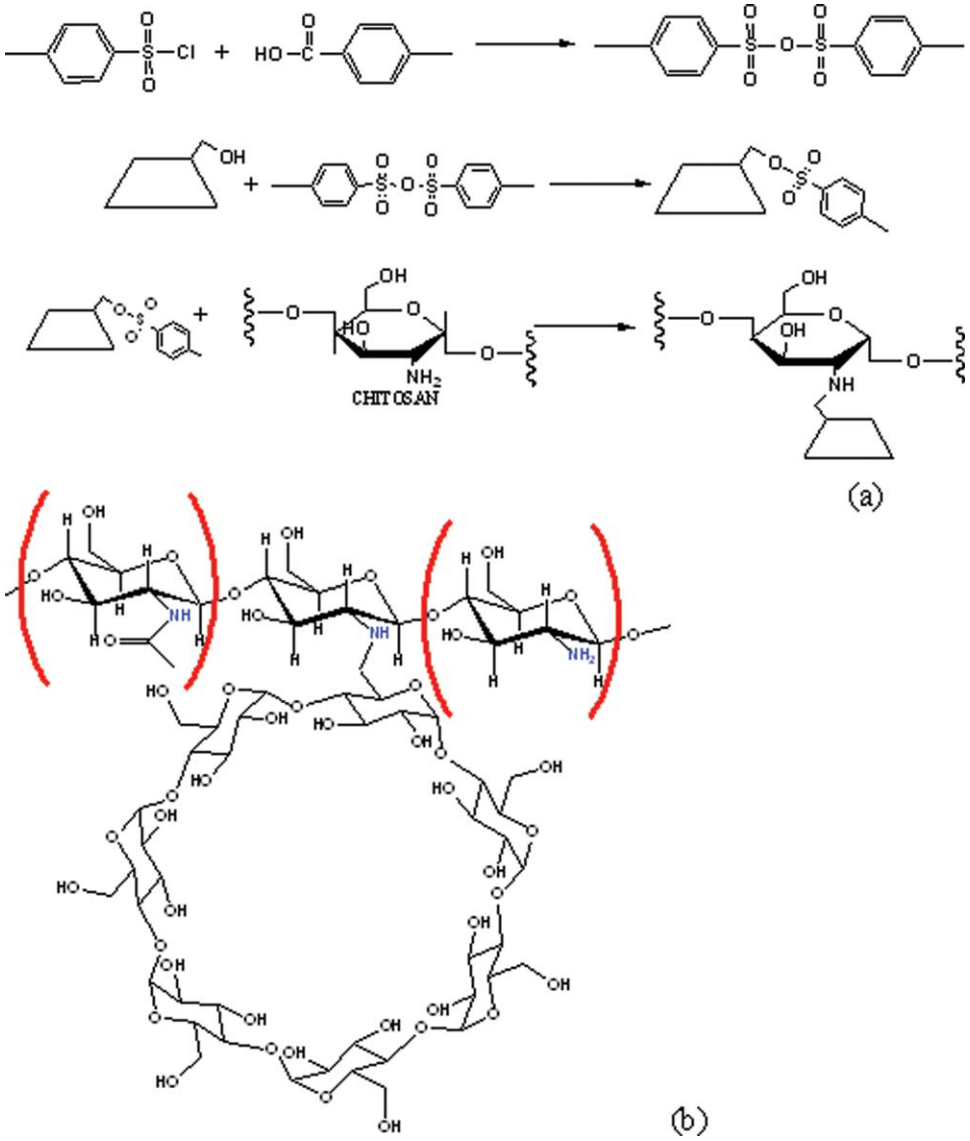

Figure 1 (a) Reaction scheme for the synthesis of β-cyclodextrin grafted onto chitosan; (b) Molecular structure of acetylated β-cyclodextrin-linked chitosan used to characterize the percentage of cyclodextrin attached to chitosan. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

bands (PB) and reference bands (RB) (A_{PB}/A_{RB}) obtained for each sample corresponded to the average value of two spectra.

Thermogravimetric analysis (TGA)

The thermal degradation of the product β-CD-chitosan was characterized by thermogravimetric analysis using a Shimadzu, TA-50 system equipped with a processor and a TA analysis program. Thermograms were measured in the range of 25–300°C at a scan rate of 10°C/min under nitrogen atmosphere.

Polymers self-aggregation

Fluorescence spectroscopy. The aggregation behavior of β-CD-linked chitosan in aqueous media was determined by fluorimetry in the presence of pyrene as hydrophobic fluorophore guest. There are five peaks in the emission spectra of pyrene in solution. The intensity of the first peak (372 nm) and the third peak (353 nm) are quite sensitive to the polarity of the microenvironment. Thus, we used pyrene as a probe to monitor the behavior of our polymers as a function of their concentration. The results obtained for β-CD linked chitosan were compared with those for unmodified chitosan.

Polymer solutions were prepared by weighing the component and stirring for 24 h in 0.5*M* CH_3COOH. Samples were protected from light to minimize photodecomposition.

For fluorescence measurements, different solutions of chitosan and β-CD linked chitosan were prepared. Pyrene previously solubilized in ethanol was added

up to a concentration of 10^{-6} M. This assay was carried out by spectrofluorimetric titration in water at pH value of 5.6. The pyrene spectra were acquired with a Thermo Spectronic Aminco Bowman (series 2) spectrofluorimeter. The excitation wavelength was 337 nm and the emission wavelength was monitored over a range of 360–600 nm (1-nm slits width). Measurements were performed at a temperature of 37°C.

Rheological characterization of crosslinking process

Chitosan and its chitosan derivative were dispersed in acetic acid solution (0.5*M*) at a concentration of 1.5%. The crosslinking process of these two polymers was characterized in the presence of genipin at 1 and 10 m*M* and at two different temperatures: 37 and 50°C.

The temperature dependence of the storage and loss moduli (G' and G'', respectively) of the aqueous dispersions was recorded in a Rheolyst AR1000*N* rheometer (TA Instruments, New Castle, DE) equipped with an AR2500 data analyzer, fitted with a Peltier temperature control, and a steel cone of 2.1° and 6 cm diameter using an angular frequency of 0.1 rad/s.

Microspheres preparation and characterization

Polymer dispersions were prepared by dissolving chitosan and β-CD-chitosan derivative (0.5%) in acetic acid (0.5*M*). Genipin was incorporated at a concentration of 1 m*M*. Microspheres were prepared by spray-drying (Mini Spray-Dryer 191) at a feed rate of 3–4 mL/min, at inlet and outlet drying air temperature of 90 and 70°C, respectively, and a pressurized air flow rate of 600 L/h. MS obtained were collected and dried under reduced pressure (20 mbar) for 24 h. The final product was stored under dry conditions at 4°C.

Particle size distribution

Microparticles were sized by laser light diffractometry using Micro Zetasizer, (Malvern Instruments, Malvern, UK). Each batch was measured in triplicate.

The average particle size distributions were expressed as the volume-weighted mode in μm and characterized by their 10%, 50%, and 90% undersize diameters, i.e., d(v, 0.1), d(v, 0.5), d(v, 0.9). The 50% undersize diameter was considered as mean diameter.

Zeta potential measurement

Spray-dried chitosan/genipin and CD-chitosan/genipin MS (0.05%; wt/vol) were prepared by dispersing the MS in KCl solution (1 m*M*). The zeta potential of MS was carried out using Laser Doppler Anemometry (Zetaplus, Brookhaven Instruments Corp.)

Scanning electron microscopy (SEM)

The shape and surface characteristics of spray-dried microparticles were observed by SEM. Samples of MS were placed on a double-sided tape previously fixed to an aluminum stage and analyzed after gold sputtering in an argon atmosphere using a Biorad E500 coating unit. Coated samples were examined using a LEO-435VP (Leica Microsystems, Cambridge, U.K.) scanning electron microscope.

RESULTS

Characterization of β-cyclodextrin-linked chitosan

In this work, the preparation and characterization of β-CD linked chitosan is described. β-CD-chitosan was successfully synthesized by the tosylation reaction. The scheme of the chemical structure of the new chitosan derivative is given in Figure 1(a). The synthesis of CD-chitosan polymers, which have been reported in other studies,[7,9,11,15,17,18] has achieved the advantages of these natural polysaccharides such as biodegradability, cytocompatibility or bioadhesion. The resulting product obtained by this method was found to be fairly nonsoluble in both neutral and acidic media (<0.1% w/v), in contrast with chitosan, which is soluble only in acidic media (>0.5% w/v). Furthermore, CD-linked chitosan was found to be nonsoluble in methanol, dimethylsulfoxide (DMSO), or DMF, with or without acetic acid.

Elemental analysis

The final product (CD-chitosan polymer) was characterized by elemental analysis. For a better comparison, chitosan and β-CD-chitosan derivative were acetylated using the same process for both compounds to enhance their solubility in solvents [Fig. 1(b)]. The products were characterized by elemental analysis of nitrogen content. The theoretical result of the calculation of molecular weight of each fragment is for original chitosan ($C_6H_{11}NO_4$:): C, 44.72%; H, 6.88%; N, 8.69%; O, 39.71% and for cyclodextrin-chitosan ($C_{48}H_{79}NO_{38}$): C, 45.11%; H, 6.23%; N, 1.10%; O, 47.57%. Therefore, the final structure should be given by the molecular formula: $[C_6H_{11}NO_4]_x[C_8H_{13}NO_5]_y[C_{48}H_{79}NO_{38}]_z$. FTIR was investigated to determine the ratio, y, of acetylated fragment. A significant reduction in nitrogen content was observed when comparing the final product (7.48%) with 44% acetylated original pure chitosan (see below) (7.90%). Thus, the total nitrogen content of the final product corresponded to a substitution degree of about 6%. Experimental elemental analysis values were C 45.78%, H 7.23%, N 7.48%, and O 39.53%. This result agreed, within a 5% difference for each component, with the theoretical value of

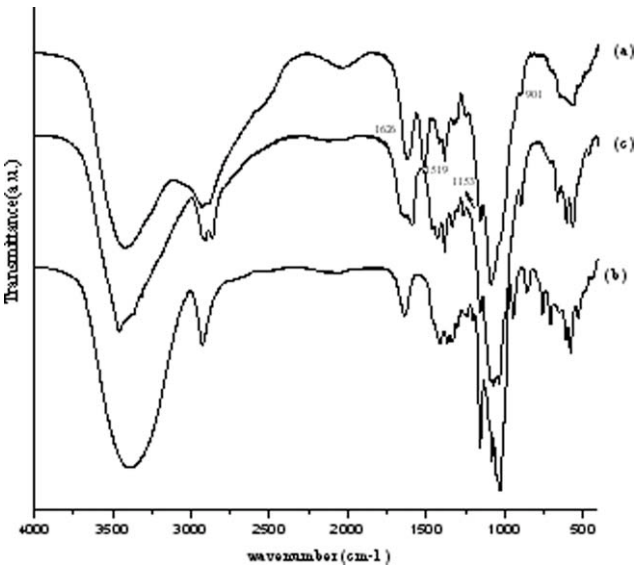

Figure 2 FTIR spectra of (a) chitosan, (b) β-CD, and (c) β-CD-linked chitosan (see also Table II).

$[C_6H_{11}NO_4]_{0.44}[C_8H_{13}NO_5]_{0.50}[C_{48}H_{79}NO_{38}]_{0.06}$, C 46.47%, H 7.57%, N 7.18%, and O 38.77%. Therefore, in the light of data obtained, the final molecular formula obtained for the β-CD-chitosan polymer was $[C_6H_{11}NO_4]_{0.94}[C_{48}H_{79}NO_{38}]_{0.06}$.

FTIR characterization of the CD-chitosan polymer

The final product was characterized by comparative FTIR analysis of acetylated chitosan and acetylated β-CD-chitosan, thereby confirming the grafting of CD onto chitosan.

IR spectra of chitosan showed the typical peaks at around 901 cm^{-1} and 1153 cm^{-1}, corresponding to the saccharide structure (Fig. 2, see also Table I for peak assignments). The strong peaks characteristic of the protonated amino group and amide I band from N-acetylation were found at 1519 and 1626 cm^{-1}. Pure CD IR spectrum revealed strong absorption bands at 3500 and 1030 cm^{-1} attributed to —OH and —C—O— groups, respectively. The spectrum of β-CD-chitosan polymer showed the characteristic strong peak at 1030 cm^{-1} of α-pyranil vibration of β-CD and the 901 cm^{-1} band for chitosan. The absorption peak at 1626 cm^{-1} due to the carbonyl stretching of secondary amide band also confirmed the grafting of β-CD onto chitosan.

The determination of the degree of acetylation from both chitosan and CD-chitosan dictated the behavior of these polymers, namely the poor solubility of this new polymer. Figure 2 shows the IR spectra of original chitosan, CD and the new polymer β-CD-chitosan. The absorbances of PB and RB were determined by the baseline method[16,19,20] (Fig. 3). The most commonly used probed bands (PB) were the carbonyl stretching and the amine bending, which clearly change their intensities with the deacetylation. In our case, the absorbance at 1557 cm^{-1} corresponding to NH bending-δ_{NH}-amide II was considered a reasonably good PB for highly acetylated samples. Three CO stretching, ν_{CO}, at 1153, 1066, 1024 cm^{-1} were analyzed and were proposed as RB. Finally, the band at 897 cm^{-1} was used as RB since it was clearly assigned.

The ratios $\frac{A_{1560}}{A_{1153}}$, $\frac{A_{1560}}{A_{1024}}$, $\frac{A_{1560}}{A_{1066}}$, $\frac{A_{1560}}{A_{897}}$ indicated an acetylation degree of 46,4, 42.6, 48.5, and 39.4%, respectively. Thus, the value for acetylation degree, AD, was calculated as 44 ± 4%.

TGA analysis

Thermogravimetric analysis is a simple method to study the decomposition pattern and the thermal stability of polymers. The early weight loss for both polymers was associated to the hydrogen bound water loss. The original polysaccharide presented its main decomposition at 234°C with a maximum weight loss of 25% of the total mass (data not shown).

Thermograms (TGA) of β-CD-chitosan revealed a main decomposition peak at 243°C in agreement with the chemical linking of CD and chitosan. There was no evidence of the existence of free cyclodextrin in the final product (CD starts to decompose at temperatures between 290 and 300°C).[21,22] In conclusion, the cyclodextrin units linked to chitosan resulted in a grafted polymer with a higher thermal stability than the pure chitosan.

Pyrene with β-CD-linked chitosan

To study the formation of aggregates at molecular level of chitosan and modified chitosan, a fluorescence spectroscopy with pyrene as a probe was

TABLE I
Main Peak Assignments in FITR Spectra for Chitosan, N-Acetylated Chitosan, β-Cyclodextrin, and N-Acetylated β-CD-Chitosan Polymer

Sample	Peak position (cm^{-1})	Peak assignment
N-Acetylated chitosan	901	Aliphatic aldehydes
	1159, 1074 and 1025	CO stretchings, ν_{CO} (alcohols)
	1519	NH bending, ν_{NH}(amide II)
	1626	Carbonyl stretching, $\nu_{C=O}$ (amide I)
	2800	CH stretchings, ν_{CH}
	3500	OH stretchings, ν_{OH}
β-Cyclodextrin	1000–1260	—C—O— stretching
	3500	—OH
N-Acetylated β-CD-chitosan	901	Aliphatic aldehydes
	1030	α-pyranil vibration (-OH)
	1626	C=O stretching of amide I

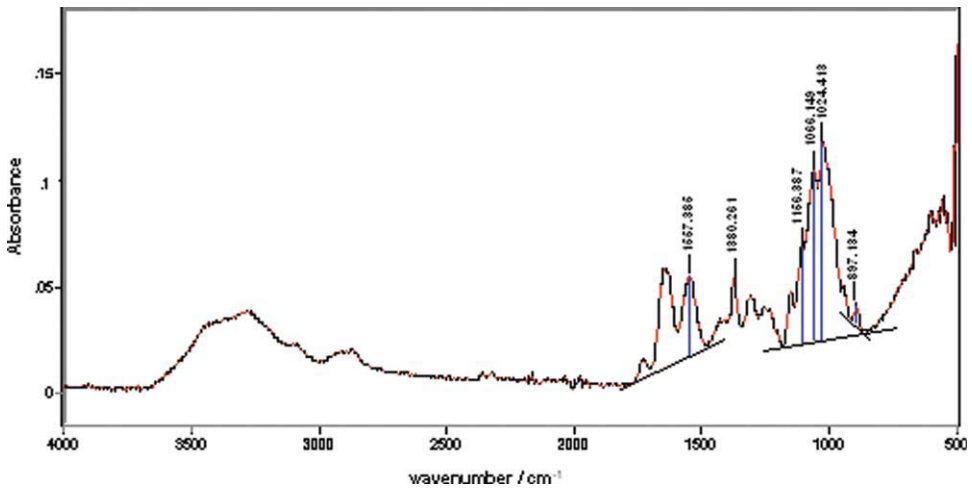

Figure 3 IR spectra of β-CD-linked chitosan with the acetylation degree of (44 ± 4)%. The intensity of maxima of the IR absorption band was determined by the baseline method. Respective baselines presented for determining DDA, 1157 cm^{-1} is the probe band and 1156 cm^{-1}, 1066 cm^{-1}, 1024 cm^{-1}, and 897 cm^{-1} are reference bands. IR measurement was carried out as KBr disk. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

used. The hydrophobic index I/III, was calculated as the ratio of the intensities at the first (372 nm) and the third (383 nm) vibronic peaks in the fluorescence spectra of pyrene. This ratio is quite sensitive to any change in the polarity of the microenvironment of the probe.[23] This is due to the fact that the fluorescent probe (hydrophobic) will preferably lie close to (or inside) the hydrophobic microdomains which are formed in a polar medium. In this case, the total fluorescence intensity increases at a certain concentration of the amphiphilic polymers, mainly due to the drastic increase of the third vibrational band. On the opposite, if hydrophobic domains are not formed in aqueous solution, the pyrene will be quenched in the polar medium. Thus, the polarity parameter, namely, I/III, is extremely sensitive to the polarization of the environment around pyrene. Fluorescence spectra of pyrene in chitosan solution or its derivative are shown in Figure 4(a,b), respectively. First, it was found that at similar concentrations, I/III values from pyrene's spectra in the presence of β-CD-chitosan polymer were significantly lower from those of pyrene in the presence of chitosan. This fact suggested the presence of hydrophobic domains in the chitosan derivative dispersion. To estimate the critical aggregation concentration, the interception of two straight lines versus polymer concentration was chosen. It was found that hydrophobic aggregates of original chitosan started at polymer concentrations of 0.83%. In the case of β-CD-linked chitosan, the presence of hydrophobic microdomains of self-aggregates in aqueous media was evident at polymer concentrations of 0.25%. At low concentrations of polymer the ratio I/III is close to that of pyrene in water for both polymers. Gradually, the polarity parameter decreased until values were considered characteristic for pyrene in hydrophobic environment in some polymers.[24,25] The emission intensity ratio I/III, with both polymer concentrations increasing, was gradually decreased and thus a hydrophobic environment for pyrene was formed.

The values of the aggregation of both the native and modified chitosan polymers clearly showed that the hydrophobic domains were formed in the original polymer. But in this case, the hydrophobic domains capable of solubilizing pyrene started to appear at much higher chitosan concentrations than in β-CD-linked chitosan [Fig. 4(b)], which showed lower polarity parameter.

The formation of hydrophobic aggregates on chitosan and modified chitosan was accompanied by a pronounced increase in the viscosity of aqueous solutions of the polymer. However, the formation of aggregates in both polymers does not lead to any precipitation. Solubility studies were also carried out using β-CD alone (data not shown), but compared with the parent and its ability to solubilize pyrene by the parent β-CD, the role of grafted β-CD is difficult to elucidate mainly due to the low concentration of CD in the grafted polymer at the concentrations used for these experiments. In comparison, in the studies with cyclodextrin alone, concentrations reached $1.3 \times 10^{-3}M$. In summary, the presence of cyclodextrin grafted to polymer resulted in a significant decrease in the critical aggregation concentration which may be indicative of certain capability for solubilizing compounds.

Rheological characterization of the crosslinking process

The rheological properties of formed hydrogel, both chitosan and chitosan modified with β-CD, were examined. β-CD-linked chitosan showed higher

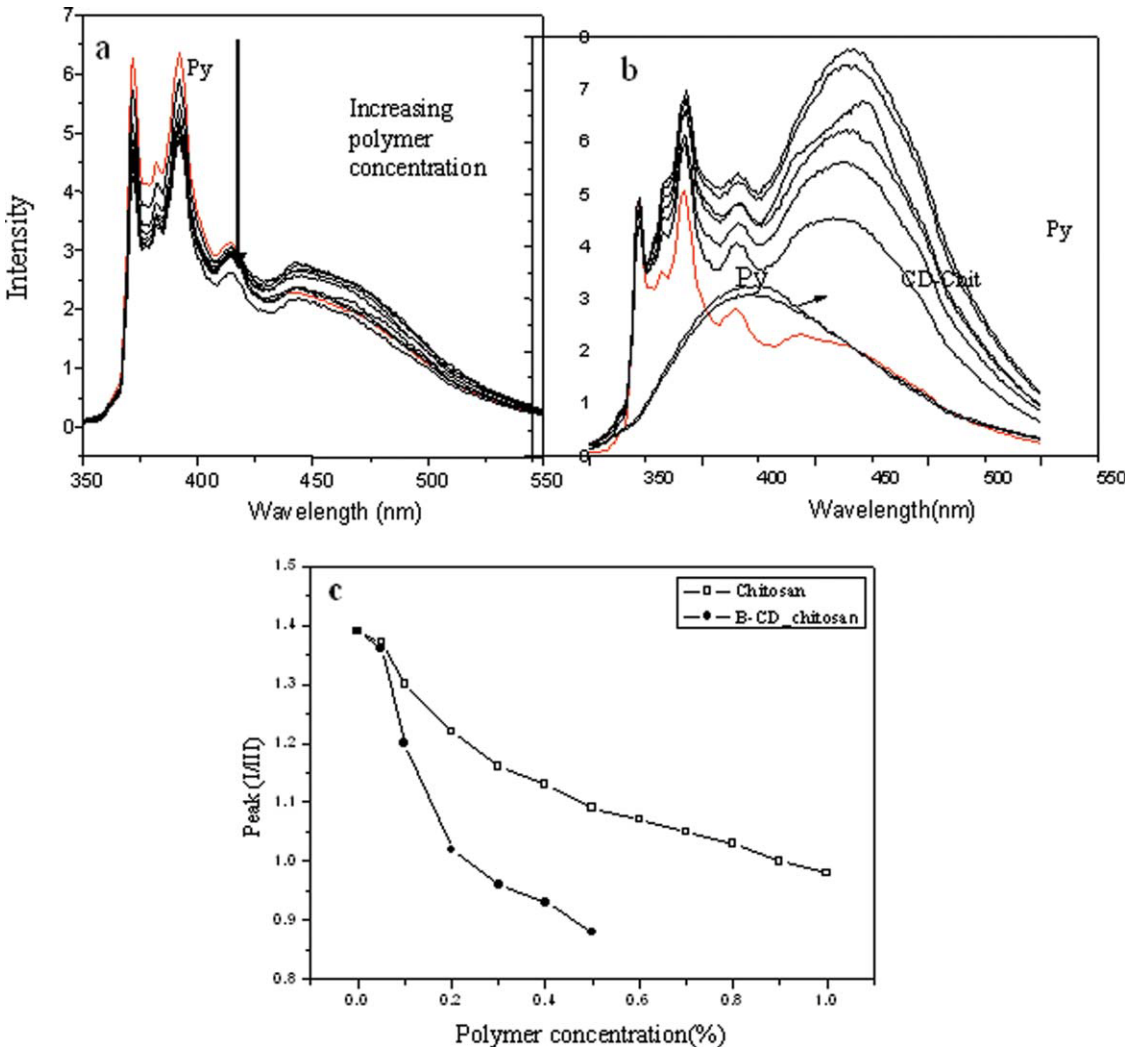

Figure 4 Fluorescence spectra of pyrene (1×10^{-6} mol L^{-1}) as a function of chitosan (a) and β-CD-chitosan derivative concentrations (0.05; 0.1; 0.2; 0.3; 0.4; 0.5%, from the base to the top) (b). The peak I/III ratio of pyrene fluorescence as a function of chitosan and β-CD-chitosan concentration. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

elastic moduli (G') and viscous moduli (G'') values than original chitosan owing to β-CD-chitosan's higher molecular weight (Fig. 5). Also, the effect of genipin concentration (chitosan crosslinker) and temperature on gelation rate and the elastic strength was investigated. The degree of crosslinking was concentration-dependent for both polymers.[26] The evolution of the elastic (G'') and viscous (G'') moduli, recorded in time-sweep experiments carried out at a fixed angular frequency (0.1 rad/s), showed that the crosslinker required a certain time to become active depending on its concentration at the temperature used as shown in Figures 6 and 7. Experiments performed at 37°C and varying the concentration of genipin for both polymers showed exactly the same pattern as was shown here for 50°C. For low concentrations of genipin (1 m*M*), an initial induction time was required, which was followed by a sharp

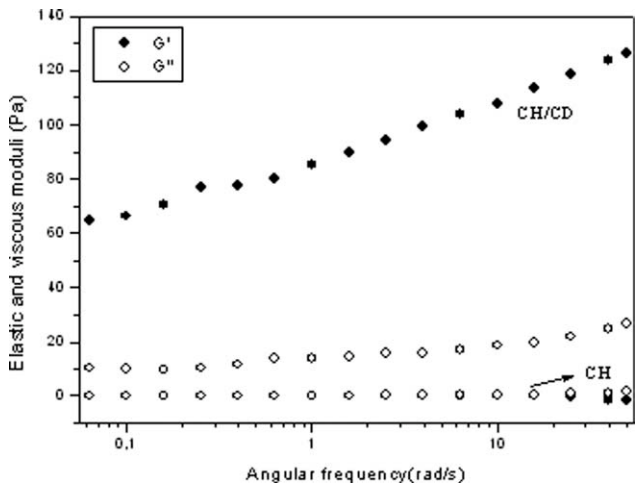

Figure 5 Viscoelastic behavior of 1.5% aqueous dispersions of original chitosan and β-CD-chitosan derivative at 37°C (angular frequency of 0.1 rad/s).

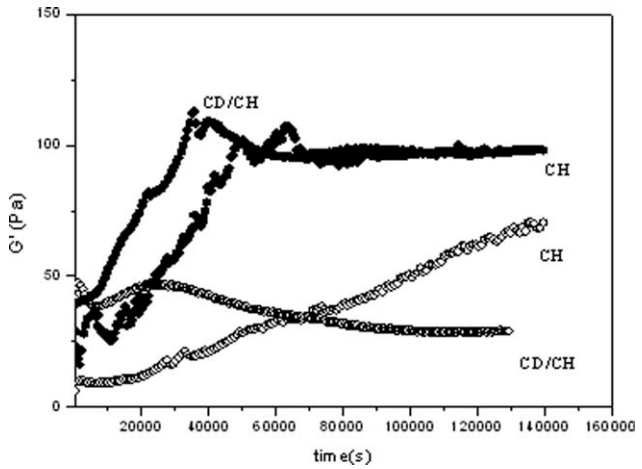

Figure 6 Viscoelastic behavior of 1.5% dispersions of chitosan and β-CD-chitosan derivative in the presence of 1 m*M* of genipin at 37°C(○) and 50°C (●). Angular frequency used: 0.1 rad/s.

increase of G'. This effect was more pronounced at higher temperatures. When the temperature of the experiments was increased from 37 to 50°C, this previous induction time decreased independently of the two concentrations of genipin investigated for both polymers, and apparently the G' values reached a plateau region within the t time scale of the experiments. However, it was observed that for the reaction between genipin and chitosan, long times are required. It is known that oxygen plays an important role in the crosslinking process.[27] Since the oxygen ingress was avoided during experiments, the reaction between genipin and polymers was even more retarded in these experiments. As previously stated, the speed and extent of the reaction between chitosan and β-CD-chitosan with genipin is clearly dependent on reaction temperature but they showed some differences in their behavior. First, the crosslinking process took place faster for β-CD-chitosan than for chitosan at the same temperature and concentrations of genipin investigated. Second, it is clearly seen that G' values reached 50-fold for β-CD-chitosan while these values are 100-fold for chitosan alone. Previous studies have demonstrated that genipin reacts with substances containing primary amino groups such as chitosan and proteins and peptides to give covalently crosslinked materials. This reaction took place by involving two different places in the genipin molecule.[28,29] Genipin can react spontaneously with amino acids or proteins by means of a ring-opening reaction to form an intermediate aldehyde group due to the nucleophilic attack by the amino groups in chitosan. A heterocyclic compound of the genipin-crosslinked chitosan is formed, followed by the opening of the dihydropyran ring and attacked by the secondary amine group on the resulting aldehyde group.

In our experiments, the increase in G' was less rapid for the β-CD-chitosan samples after mixing the polymer with genipin than the increase achieved for the original chitosan reacting with genipin. Also, lower values of G' were reached for modified chitosan with β-CD compared with those values for original chitosan. This finding could be due to the lower number of amine residues available in β-CD-chitosan for reacting in the crosslinking reaction in this system. Since genipin molecules were reacting in two steps leading to the formation of crosslinks between primary amino groups,[28] the presence of β-CD groups in the modified chitosan delayed the crosslinking reactions because genipin molecules were sterically impeded from being linked.

Particle size distribution, ζ-potential, and morphology of MS (SEM)

β-CD-chitosan MS presented a particle size of an average of 9.93 ± 0.8 μm, which was smaller that the particle size determined for the chitosan microparticles: 26.09 ± 0.5 μm. This finding might be attributable to lower water uptake by β-CD-chitosan microparticles during the measurement, which is due to their lower polarity exhibited by the grafted polymers, as fluorescence spectroscopy studies showed. For both polymers, the presence of genipin gave lower particle size. The ξ-potential of MS produced by spray-drying is compiled in Table II. Grafting the cyclodextrin to chitosan led to a significant modification of positive ξ-potential (+40.4 to + 28.1 mV), justified by the masking of free positively charged amino groups of chitosan. In both cases, crosslinking of MS using 1 m*M* genipin slightly decreased the values of surface particle charge, indicating a certain

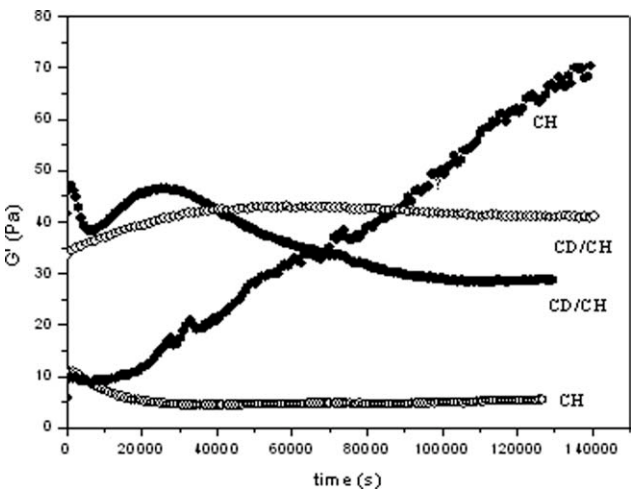

Figure 7 Effect of concentrations of genipin of 1 m*M* (○) and 10 m*M* (●) on the evolution of elastic modulus for chitosan and β-CD-chitosan, both at 50°C; angular frequency 0.1 rad/s.

TABLE II
Mean Size and ζ-Potential of Microspheres Obtained by Spray-Drying (n = 3 ± s.d.)

	Chitosan MS	B-CD-chitosan MS	Cross-linked Chitosan MS	Cross-linked β-CD-Chitosan MS
Size	26.09 ± 2.56	9.93 ± 0.82	10.35 ± 1.89	3.09 ± 0.52
ζ-potential	40.41 ± 0.57	29.54 ± 0.77	28.39 ± 1.02	22.39 ± 1.11

degree of reticulation, but the zeta potential remained positive. These results indicated that the inherent properties of chitosan, such as adhesion onto biological tissues (mucus, gastrointestinal tract) and its ability as penetration enhancer and interaction with cells, were maintained despite the presence of cyclodextrin.

The microparticles obtained from β-CD grafted onto chitosan aqueous polymer solution were characterized by SEM to visualize their shape and surface. Micrographs revealed a smooth surface and spherical shape for β-CD-chitosan microparticles (Fig. 8). As shown, particles made of unmodified chitosan resulted in a non spherical shape and a very irregular surface. This effect was observed for MS made of chitosan independently of the presence of genipin.

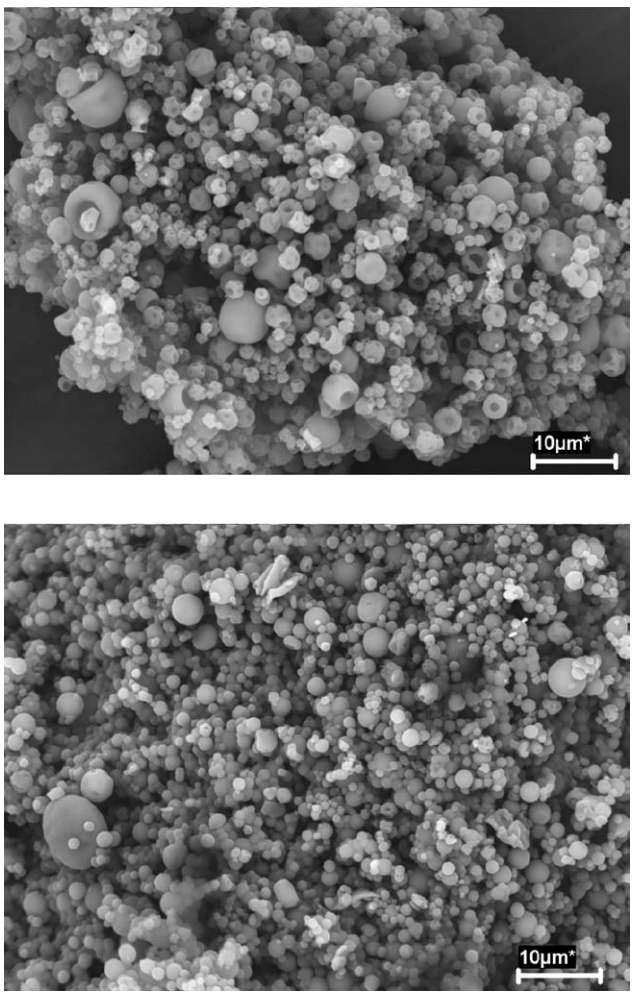

Figure 8 SEM pictures of microparticles made of (a) 1% chitosan crosslinked with 10 m*M* genipin and (b) 1% β-CD-chitosan crosslinked with 10 m*M* genipin.

FTIR characterization of crosslinked MS

Figure 9 shows the IR spectra of chitosan and β-CD-chitosan microparticles crosslinked by genipin at 10 m*M* after spray-drying. Noncrosslinked microparticles made of chitosan showed the same characteristic peaks for chitosan already described above. Comparison of the IR spectrum of chitosan microparticles and chitosan microparticles crosslinked with genipin showed some pronounced differences. The appearance of a peak with stronger adsorption at 1626 cm^{-1} assigned to the amide groups of chitosan, and the decreased adsorption at 1570 cm^{-1} which is associated to the amino groups, might be attributed to the formation of the secondary amide as a result of the reaction between amino groups of chitosan

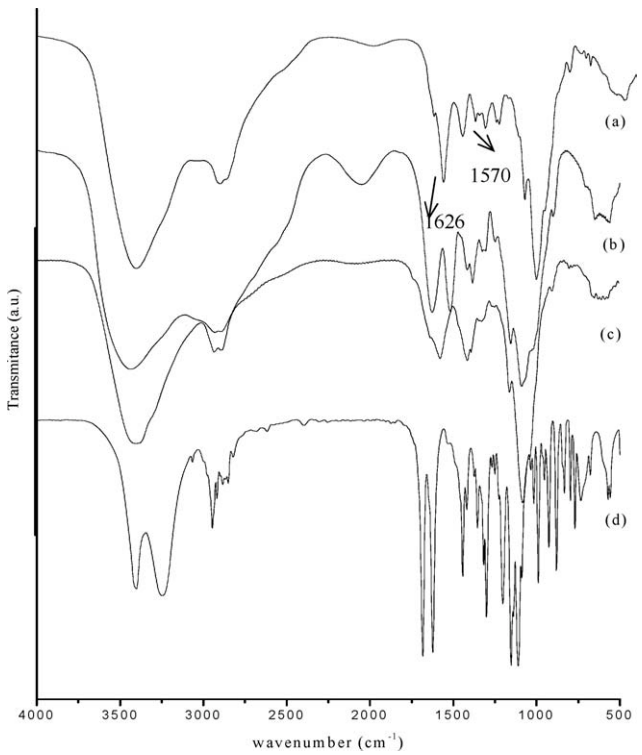

Figure 9 FTIR spectra of chitosan crosslinked with genipin microparticles (a), plain chitosan microparticles (b), β-CD-chitosan crosslinked with genipin microparticles (c), and pure genipin (d).

and ester groups of genipin. This evidence of covalent crosslinking could not be found for the new polymer β-CD-chitosan due to the overlapping of peaks adsorption in the IR spectrum.

CONCLUSIONS

CD chitosan derivative was synthesized by reacting chitosan with tosylate. The final chitosan derivative exhibited CD moities in 6% of glucosamine units. Elemental analysis and FTIR confirmed the formation of an amide linkage between chitosan and cyclodextrin. These studies provided clear evidence of CD grafting degree of approximately 6%. The fluorescence spectroscopy showed the formation of hydrophobic local domains in β-CD-linked chitosan at lower concentrations from native chitosan. MS were prepared with the new polymer by spray-drying and crosslinked with genipin. The particles obtained with the β-CD-chitosan polymer showed homogeneous size distributions, smooth and regular surfaces and positive values for ζ-potential. As rheology results showed, crosslinking with genipin was temperature and concentration-dependent for chitosan and β-CD-linked chitosan. These results illustrate the possibility that β-CD-linked chitosan MS might serve as a carrier for drugs in controlled drug release. β-CD-chitosan polymer is also not costly and can be easily prepared. The chemical modification of chitosan with β-CD introduced novel features to this biopolymer such as hydrophobicity which combined with its known biocompatibility and the enzymatic biodegradability of chitosan and CD makes this new grafted polymer a promising alternative for hydrophobic drug encapsulation. This microparticulate system might increase the absorption of poorly soluble drugs and sustain the release of certain drugs for prolonged times.

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