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Biodegradable chitosan nanogels crosslinked with genipin

Maite Arteche Pujana, Leyre Pérez-Álvarez*, Luis Carlos Cesteros Iturbe, Issa Katime

Grupo de Nuevos Materiales y Espectroscopia Supramolecular, Departamento de Química Física, Facultad de Ciencia y Tecnología, Universidad del País Vasco (UPV/EHU), 644 Bilbao, Spain

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ABSTRACT

Chitosan nanoparticles crosslinked with genipin were prepared by reverse microemulsion that allowed to obtain highly monodisperse (3–20 nm by TEM) nanogels. The incorporation of genipin into chitosan was confirmed and quantitatively evaluated by UV–vis and ^{1}H NMR. Loosely crosslinked chitosan networks showed higher water solubility at neutral pHs than pure chitosan. The hydrodynamic diameter of the genipin–chitosan nanogels ranged from 270 to 390 nm and no remarkable differences were found when the crosslinking degree was varied. The hydrodynamic diameters of the nanoparticles increased slightly at acidic pH and the protonation of ionizable amino groups with the pH was confirmed by the zeta potential measurements. The biocompatible and biodegradable nature, as well as the colloidal and monodisperse particle size of the prepared nanogels, make them attractive candidates for a large variety of biomedical applications.

1. Introduction

Chitosan is a linear and partly acetylated (1–4)-2-amino-2-deoxy-β-D-glucan isolated from marine chitin (Muzzarelli, Boudrant, et al., 2012; Muzzarelli, Jeuniaux, & Gooday, 1986). In contrast to other polymers, chitosan is positively charged due to the protonation of its primary amines weak basic groups, which give it special characteristics and feasibility to chemical modification. Chitosan exhibits many favourable characteristics such as biodegradability, nontoxicity and biocompatibility, which give it a great potential in a wide range of areas such as food, cosmetics, wastewater treating, biotechnology, or biomedicine (Crini, 2005; Ravi Kumar, 2004; Shahidi, Arachchi, & Jeon, 1999).

Chitosan has been extensively studied for delivery of bioactive reagents, including drugs (Felt, Buri, & Gurny, 1998), proteins and genes (Malhotra et al., 2009) and it is, in this respect, one of the preferred candidates in advanced fields like gene delivery (Liu & Yao, 2002), transnasal delivery (Illum, Farraj, & Davis, 1994) or ocular diseases treatment (Alonso & Sánchez, 2003). Regarding this purpose chitosan has been prepared in a wide variety of forms, namely hydrogels, beads, films, tablets, microspheres, composites, micro and nanoparticles (Banerjee, Mitra, Kumar Singh, Kumar Sharma, & Maitra, 2002; Lee et al., 2002; Miyazaki, Nakayama, Oda, Takada, & Attwood, 1994).

Nanoparticles have a special role in targeted drug delivery due to their long shelf life, easy transport to different body sites and high drug entrapment efficacy (Agnihotri, Mallikarjuna, & Aminabhavi, 2004). Chitosan nanoparticles can be obtained by chemical crosslinking leading to quite stable matrixes. These chitosan nanogels are commonly prepared by modification with bifunctional agents such as diisocyanate, diepoxy compounds, dialdehyde and other reagents (Rinaudo, 2010; Wenling et al., 2005). Among them, in the last decades glutaraldehyde was the most broadly used crosslinker agent in covalent formulations. However, due to the proved toxicity of glutaraldehyde, recently, biocompatible crosslinking agents have received much attention in the field of biomedical application.

Extended studies about genipin, an alternative natural crosslinking agent, has shown its ability to form biocompatible and stable crosslinked products 10,000 times less cytotoxic than glutaraldehyde (Sung, Huang, Huang, & Tsai, 1999). Genipin is an iridoid glucoside extracted from Gardenia that has been used in traditional Chinese medicine and as a blue colourant in the food industry.

Genipin reacts under mild conditions with primary amine groups and this reaction, which is undoubtedly identified by mean of its inherent blue colouration, has been employed to crosslink chitosan (Jin, Song, & Hourston, 2004) and other amino group containing biomaterials (González, Strumia, & Alvarez Igarzabal, 2011).

Genipin has been widely employed in the field of biomaterials specially in studies of tissue fixation and regeneration in various biomaterials and natural biological tissues, or in studies of drug delivery of macrogels, microspheres and semi-interpenetrating polymer networks (semi-IPNs) (Muzzarelli, Greco, Busilachi, Sollazzo, & Gigante, 2012). Additionally, genipin has been employed to obtain nanocomposites consisted of metal nanoparticles

* Corresponding author. Tel.: +34 946012711; fax: +34 946013500.
E-mail address: leyre.perez@ehu.es (L. Pérez-Álvarez).

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embedded in chitosan crosslinked matrixes (Liu & Huang, 2008). However, there are not many publications relating to genipin crosslinked nanoparticles (Choubey & Bajpai, 2010; Maggi, Ciccarelli, Diociaiuti, Casciardi, & Masci, 2011) which encourages us the use of genipin as a crosslinking agent to develop chitosan nanogels for biomedical applications.

Since size distribution plays an important role in drug delivery application, it is necessary to prepare uniformly sized nanoparticles. However, the size distribution of chitosan nanoparticles is very difficult to control. Various studies have shown that reverse microemulsion (w/o) is an effective way to prepare low sized particles with uniform particle size distribution. This promising method for the effective preparation of chitosan nanoparticles is a transparent, isotropic and thermodynamically stable synthesis medium. Microemulsions not only act as microreactors for the formation of nanoparticles but also inhibit excessive aggregation of the forming particles (Liu, Shao, Gea, & Chena, 2007). Several authors have employed the well established microemulsion formulation based on cyclohexane/Triton X-100/*n*-hexane/water for obtaining chitosan nanosized particles (Arteche Pujana, Pérez-Alvarez, Cesteros Iturbe, & Katime, 2012; Wang, Wang, Luo, & Dai, 2008).

In this work chitosan and genipin were chosen for preparing polymeric nanogels by crosslinking of chitosan. Both of these compounds display advantageous biomedical properties. Nanogels were preparing by w/o microemulsion method and their physicochemical properties, such as, particle size, pH-sensitivity swelling, surface charge or water solubility, were studied.

2. Experimental

2.1. Materials

Low molecular weight chitosan (Aldrich) was purified using the procedure previously described (Signini & Campana Filho, 1999). The deacetylation degree of chitosan determined by ^{1}H NMR was 79% which is in good agreement with the value reported by the supplier (75–85%). Its viscosity average molecular weight of chitosan measured by an Ubbelohde capillary viscometer (HAc 0.3 M/NaAc 0.2 M, 25 °C) (Rinaudo, Milas, & Dung, 1993) was 66,000 g/mol. Genipin, Triton X-100 and hexanol (for synthesis, 98%) were purchased from Sigma–Aldrich. Cyclohexane (for synthesis, 98%), acetic acid (for analysis, 99.8%) and ethanol (for analysis, 96%) were supplied from Panreac and D-glucosamine hydrochloride by Acros Organics.

2.2. Preparation of nanogels

1 g of chitosan was dissolved in 100 mL of 1% (v/v) aqueous acetic acid solution and separately genipin was dissolved in distilled water (1–540 mg/mL).

Chitosan nanoparticles were prepared using a procedure that was previously described by Jia, Yujun, and Guangsheng (2005) with minor modification. Two separate w/o microemulsions were produced. The first microemulsion (MC) was formed by dropwise addition of Triton X-100 to a mixture of cyclohexane:*n*-hexanol:chitosan solution in ratio of 2.75:1:1 respectively. Triton X-100 was added until microemulsion became optically transparent. The second microemulsion (MG) containing genipin solution but without chitosan was prepared with the same procedure. Then, MG microemulsion was added to MC microemulsion under magnetic stirring at different crosslinker stoichiometric ratio. The crosslinking reaction was carried out at 25 °C for 4 days. During crosslinking reaction the microemulsion became bluish. The microemulsion was broken by adding ethanol to obtain blue coloured nanoparticles. Nanogels were washed repeatedly with ethanol by centrifugation (9000 rpm, 10 min, at room temperature). Finally prepared nanogels were washed with distilled water, ultrafiltered (OMEGA 76 MM 100 K 12/PK) and vacuum dried (55 °C, 24 h).

2.3. Methods

The nanogels were analyzed with NMR spectroscopy. ^{1}H spectra were obtained on a Bruker Avance 500 MHz instrument and the samples were dissolved in 2% (w/w). CD_3COOD/D_2O. Solid samples were analyzed by ^{13}C NMR spectroscopy with a Bruker Avance III 400 spectrometer.

A Philips CM120 with Olympus SIS Morada digital camera transmission electron microscope was used to characterize the size and morphology of the dried chitosan nanoparticles. Nanoparticles were prepared dissolving dried powder in 1% (v/v) aqueous acetic acid solution with a concentration of 1 mg/mL which was then dried in a vacuum chamber.

UV–vis spectroscopy measurements were registered in a Cintra 303 UV-vis Spectrophotometer.

The genipin–chitosan crosslinking reaction was characterized by UV–vis. Along the crosslinking reaction, spectra of the microemulsion samples were obtained at different time intervals in a wavelength range from 200 to 700 nm. Furthermore, the quantitative determination of the reacted free amino groups in nanoparticles crosslinked with genipin was carried out by mean of the previous calibration with D-glucosamine in the microemulsion medium. D-Glucosamine (50–750 mg/L) and genipin (500 mg/L) after being dissolved in the described microemulsion medium were incubated at 25 °C. The absorbance at 605 nm was measured and the obtained linear regression equation was $A = 0.0188 + 0.0021X$ ($R^2 = 0.99764$).

UV–vis spectroscopy was also employed for the study of the water solubility of the nanogels at different pH values by mean of the measurement of the transmittance of the nanogels dispersions at 750 nm. Nanogels were dissolved in 1% HAc solution (1 mg/5 mL), and the transmittance was measured 24 h after the pH of the solution was adjusted by the addition of 2 N NaOH solution.

Dynamic laser light scattering measurements for determining the size of the nanogels were performed using a Brookhaven BI-9000AT with a goniometer. A water-cooled argon-ion laser was operated at 514.5 nm as the light source. The time dependence of the intensity autocorrelation function of the scattered intensity was obtained by using a 522 channel digital correlator. The size of the nanoparticles was determined from the diffusion intensity of the particles using the Stokes–Einstein equation. The size distribution was obtained by NNLS analysis and the uncertainties of the measurements represented the standard deviation of the mean of the replicate runs. The dried powder samples were dispersed in 1% HAc solution (1 mg/mL) during 3 days and then diluted until the concentration was 40 mg/L. All measurements were made at room temperature. The pH was changed with 2 N NaOH solution.

X-ray diffraction patterns were obtained by a Transmision STOE Stadip X-ray diffractometer with a goniometer speed of 0.1°/90 s. The range of diffraction angle 2θ was 2.5–34°.

Electrophoretic mobility measurements were performed with a Zeta-Sizer IV (Malvern Instruments). Nanogels were dispersed in 1% HAc solution (1 mg/mL) during 3 days and finally diluted to a concentration of 40 mg/L. Data were obtained from the average of at least ten measurements in universal cells. The external pH was varied by adding 2 N NaOH solution.

3. Results and discussion

3.1. Study of the crosslinking reaction

Covalently crosslinked chitosan nanoparticles were prepared within a reverse microemulsion medium in order to control the

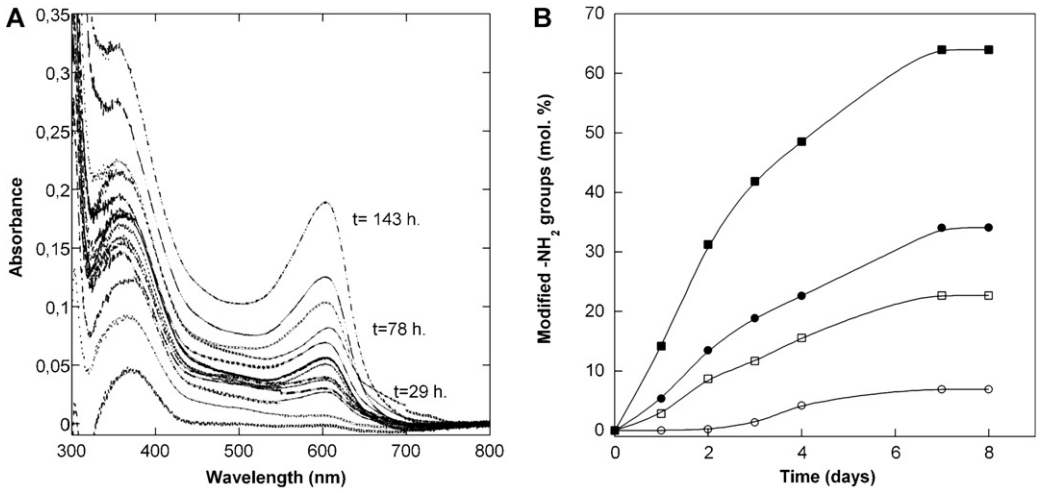

Fig. 1. Evolution of (A) the visible absorption spectrum of chitosan microemulsion during crosslinking reaction with genipin and (B) modification degree of chitosan with crosslinking reaction time for: (○) 7.5, (□) 12.5, (●) 18.8, (■) 37.5 mol.% of genipin.

colloidal particle size of the prepared nanogels. This modification reaction took place when separately prepared chitosan and genipin microemulsion were mixed as described above. This crosslinking occurs by mean of a complex mechanism which is believed that consists in two reactions involving different sites on the genipin molecule (Butler, Ng, & Pudney, 2003) and produces dark-blue colouration in the reaction mixture. The appearance of this colouration in the reaction mixture of genipin and chitosan is traditionally considered as rapid evidence that the crosslinking reaction is taking place (Butler et al., 2003; Muzzarelli, 2009).

The evolution of the crosslinking reaction was monitored by mean of the UV–vis spectra of the final microemulsion. As can be seen in Fig. 1A, a new peak appeared at 605 nm, which corresponds to the formed blue pigment, and continued to increase in intensity throughout the course of the reaction.

Ramos-Ponce et al. (2010) described an efficient colorimetric method using genipin as a specific reagent for quantitative determination of free amino groups of chitin derivatives. Following this procedure, the relationship between the absorbance of the microemulsion at 605 nm and the concentration of free amino groups in the nanogels was determined by mean of a calibration curve with standard solutions of D-glucosamine. The method reported by Ramos et al. could be applied to the microemulsion medium after slight modifications. The concentration of modified free amino groups as a function of reaction time was analyzed in order to optimize the reaction time. Results of this study are shown in Fig. 1B, demonstrating that after 7 days the end point of the reaction was reached.

3.2. NMR characterization

The success of the modification reaction was also confirmed and quantitatively evaluated by ^{1}H NMR. Pure chitosan shows a peak at 2.0–2.1 ppm corresponding to the three protons of *N*-acetyl glucosamine (GlcNAc) and a peak at 3.1–3.2 ppm due to H-2 proton of glucosamine (GlcN) residues which represent the primary amino group content of the chitosan. Fig. 2 displays the spectra of several genipin–chitosan nanogels. A clear decrease of the signal at 3.2 ppm was observed for all the samples, indicating the loss of amine groups by reaction with genipin showing that the addition of genipin to chitosan was successfully carried out.

The peak of the ^{1}H NMR spectrum of chitosan corresponding to the methyl protons at 2.0–2.1 ppm possess the highest resolution (Kasaai, 2010) and was used for the determination of the free amino content by mean of the integration of the resonance of H-2 protons at 3.2 ppm. Thus, the modification degree of the chitosan chains was evaluated by the decrease of the glucosamine signal (3.2 ppm). The crosslinking grade was calculated assuming that the reaction occurs on both reaction sites of the genipin molecule. Table 1 lists the nanogels compositions obtained by ^{1}H NMR analysis for each initial reaction feed. The data collected in Table 1 show a rather good correlation between genipin stoichiometric ratio (with respect to the total amount of glucosamine units) in initial reaction mixture and final modification degree in the nanogels, especially for intermediate compositions. These results suggest that the linkage genipin–chitosan occurs in a ratio around 1:2 and therefore the self-polymerization of genipin is not likely to take place. However, for high crosslinker content, the ratio genipin feed: modified chitosan increased. Assuming that the reaction yields did not varied significantly, it could be related to a higher probability that polymerization takes place. This in accordance with an intermediate brownish colour appeared during crosslinking reaction of these samples before the blue intense colouration (Mi, Shyu, & Peng, 2005).

On another hand, modification degrees of chitosan in the nanogels obtained by ^{1}H NMR analysis agreed with those obtained by UV–vis spectroscopy (Table 1).

Besides, ^{13}C NMR spectroscopy also corroborates the modification of linear chitosan with genipin. Fig. 3 shows the ^{13}C NMR of initial pure chitosan and chitosan crosslinked with genipin. The crosslinking of chitosan with genipin results in some slight changes on the ^{13}C NMR spectrum of chitosan. The resonance signal of chitosan C-4 at 81–87 ppm decreased and becomes into a shoulder. Mi, Sung, and Shyu (2000, 2001) attributed this fact to a conformational change from the linear structure of original chitosan to crosslinked chitosan, revealing the formation of a secondary amide and heterocyclic amino linkage, and so, the crosslinking of chitosan. Additionally, a slight shift of C-1 resonance from 112 ppm to a broad resonance centred at around 109 ppm occurs, which is attributed to the incorporation of the heterocyclic ring at C-2 (Mi et al., 2000).

3.3. Water solubility

The applications of chitosan nanoparticles suffer severe limitations because they are no dispersible in neutral or alkaline pH aqueous media. This water insolubility in high pH solutions is due

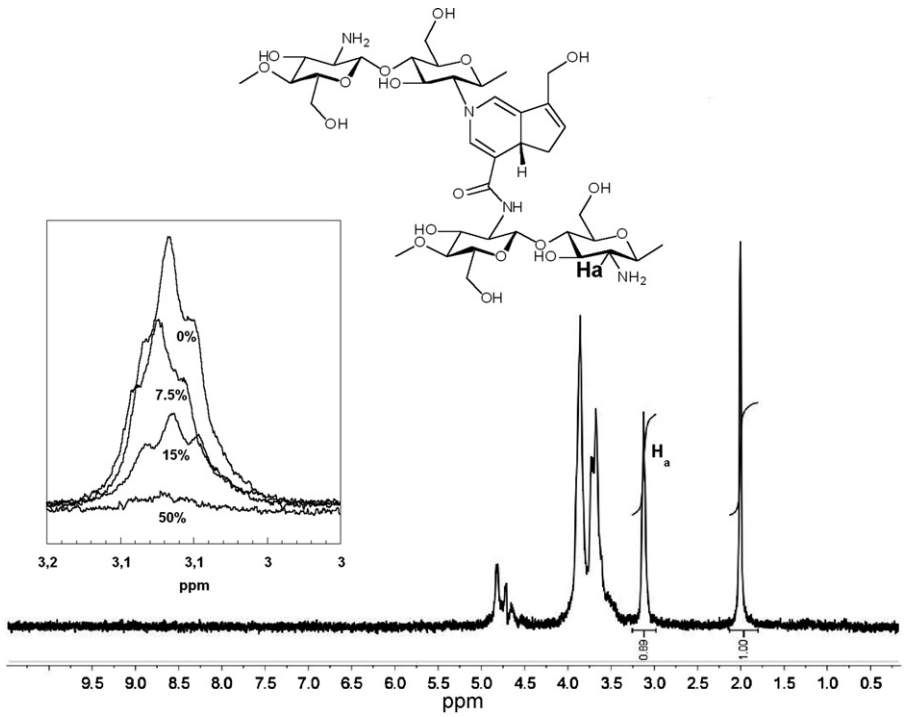

Fig. 2. ^{1}H NMR spectrum of genipin–chitosan nanogels (7.5 mol.% of genipin) and amplified resonance signal corresponding to the H_a protons (chitosan H-2 of GlcN) of nanogels crosslinked with different genipin feeds (0, 7.5, 15 and 50 mol.%).

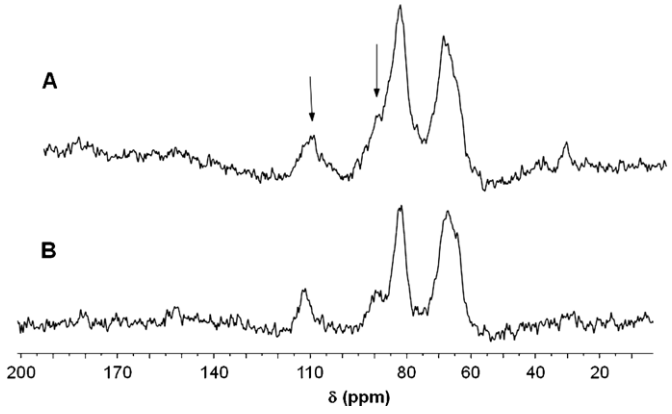

Fig. 3. ^{13}C NMR spectrum of (A) chitosan nanoparticles crosslinked with genipin and (B) pure chitosan.

to the presence of rigid crystalline domains, formed by intra- and/or intermolecular hydrogen bonding (Nishimura, Kohgo, Kurita, & Kuzuhar, 1991).

The solubility of the prepared genipin–chitosan nanogels was characterized as a function of pH and compared with the unmodified chitosan. Fig. 4A shows the transmittance at 750 nm of aqueous solutions of linear chitosan and genipin–chitosan nanogels prepared in the above-described conditions.

In general, as the pH decreases the protonation of free amino groups of chitosan chains takes place and the H-bonding interactions decreases, improving water–polymer interaction and consequently increasing the transmittance of aqueous dispersions (Fig. 4A). So, when pH increases the dispersions become opaque and particles aggregation takes places.

The solubility of the nanogels was determined from pH_{50} parameter that is defined as the pH value when the transmittance at 750 nm reached 50%. Loosely crosslinked nanogels (<7.5 mol.% genipin) showed a slightly improved stability with the pH, displaying a value of 7.3 for the pH_{50} parameter, while the value measured for pure chitosan was 7.0. When the genipin content was higher than 7.5 mol.% the pH_{50} of the nanogels was lower than that of the pure chitosan in all the cases.

It is well kwon that the modification of chitosan with more or less bulky pendant groups leads to the reduction of hydrogen bonding in chitosan and therefore, the solubility of the chitosan derivatives increases. In this regard, the increase in water solubility of PEGylated chitosan (Chan, Kurisawa, Chung, & Yang, 2007) has been attributed to the decrease of intermolecular interactions, as well as the swelling of *N*-alkyl and *N*-acylated chitosans in water, in spite of their hydrophobicity (Ravi Kumar, 2000; Shasiwa et al., 2002).

Table 1
Modification degree of obtained genipin–chitosan nanogels analyzed by ^{1}H NMR and UV–vis spectroscopy.

	^{1}H NMR		UV–vis	
Genipin Feed (stoichiometric mol.%)	Modification degree (mol.%)	Crosslinking (mol.%)	Modification degree (mol.%)	Crosslinking (mol.%)
7.5	15	7.5	7	3.5
15.0	30	15	30	15
18.8	34	17	34	17
25.0	39	20	44	22
50.0	71	36	76	38

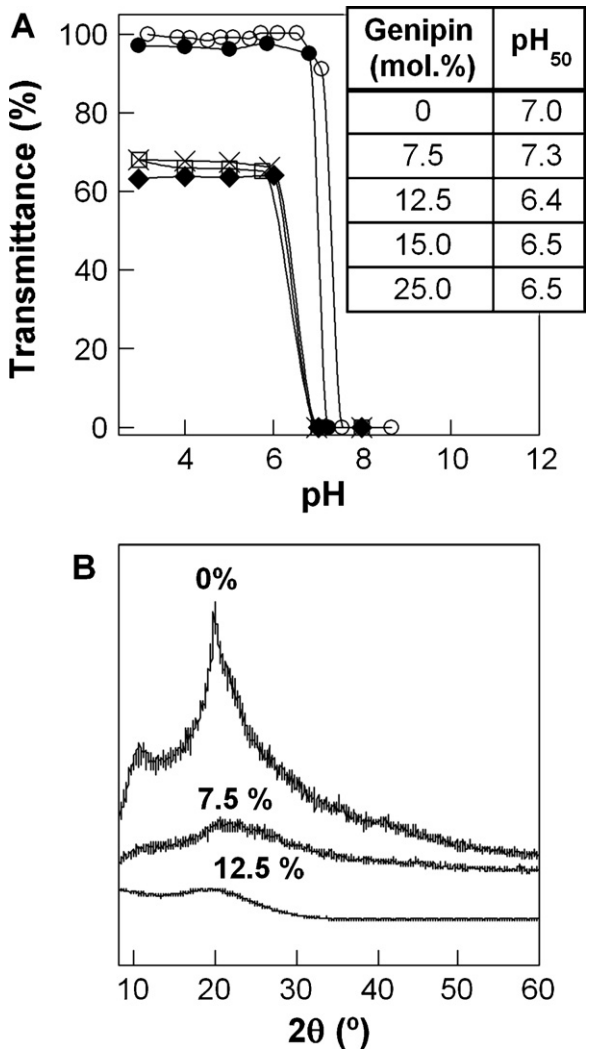

Fig. 4. (A) Transmittance variation with external pH for (●) pure chitosan and, genipin–chitosan nanogels with (○) 7.5, (□) 12.5, (♦) 15 and (×) 25 mol.% of genipin in feed (1 mg/50 mL in 1% HAc solution). (B) X-ray diffraction pattern of genipin-crosslinked nanoparticles with different mol.% of genipin.

In general, chitosan derivatives that precipitate in basic medium have a high degree of crystallinity. On the contrary, chitosan obtained with amorphous form and loosely aggregated state is soluble in water (Lu, Song, Cao, Chen, & Yao, 2004).

Fig. 4B shows the X-ray diffractograms of some of the samples whose solubility behaviour is displayed in Fig. 4A. Pure chitosan sample displays the typical pattern of chitosan substrates with peaks at 2θ around 11° and 20°. As can be observed, the chemical crosslinking with genipin causes the loss of crystallinity in the samples. All the studied genipin–chitosan nanogels displayed a diffractogram of an amorphous material.

Thus, it could be concluded that a moderate crosslinking disturbs the intra and inter intermolecular hydrogen bonding resulting in the observed enhancement in solubility. However, as crosslinking increases (up to 7.5 mol.%), in spite of the weakened intra-intermolecular interactions of chitosan chains, the nanogels become more and more compact and a close and rigid network is generated, leading to a substantial reduction of the water solubility.

3.4. Particle size and swelling properties of the nanogels

The morphology and particle size distribution of dried nanogels was studied by TEM. TEM images (Fig. 5A) indicate that genipin–chitosan nanogels are spherical, have nearly uniform particle size distribution (Fig. 5B) and there is no severe particle agglomeration, which are very interesting qualities for biomedical applications, such as drug delivery. The average diameters of nanoparticles ranged from 3 to 20 nm.

The particle size of swollen nanogels was determined by QELS measurements. Due to the adhesive nature of chitosan in aqueous solution, these nanoparticles tend to form aggregates that lead to a higher average hydrodynamic diameter than the actual size of the particles (Wu, Zhou, & Wang, 1995). The progressive dilution of the nanoparticle dispersions has been reported as an effective way to reduce the inter-particle interaction and the presence of large aggregates of chitosan (Sorlier, Rochas, Morfin, Viton, & Domard, 2003) and it was applied in this study. However, the solubility study revealed in comparison with pure chitosan a higher tendency of genipin–chitosan nanogels to aggregate, except for loosely crosslinked ones (7.5 mol.%).

Fig. 6 shows the average diameters of the nanogels determined by QELS at pH 4.0 for several genipin stoichiometric ratios. No strong correlation was found between the hydrodynamic diameter of the genipin–chitosan nanogels and the crosslinking degree. Besides, as is shown in Fig. 7, a slight pH-responsive behaviour was observed. Thus, it could be concluded that the nanogels display a weak swelling capacity due to the rigid and hydrophobic nature of the employed crosslinker.

The swelling effect induced in an ionic network, such as chitosan nanogels, can be considered as the accumulative effect of

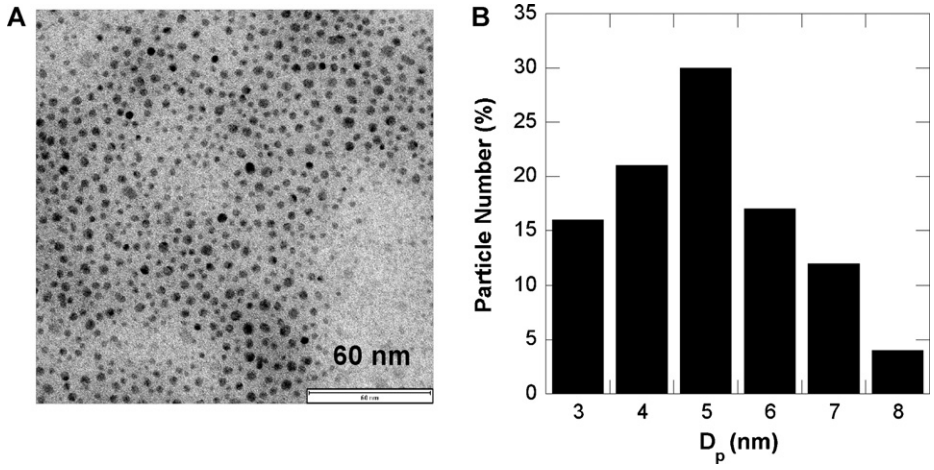

Fig. 5. (A)TEM microphotograph of genipin crosslinked chitosan nanoparticles (12.5 mol.% of genipin) and (B) particle size distribution obtained by TEM.

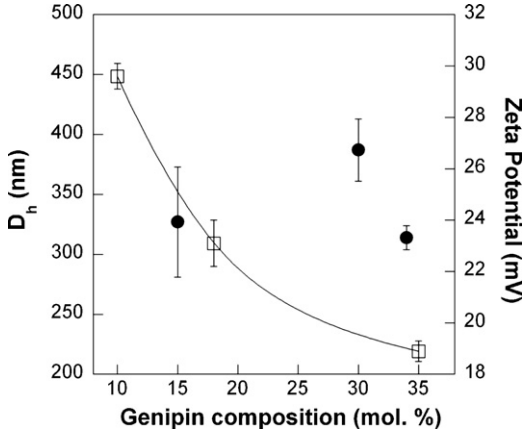

Fig. 6. (□) Zeta potential (●) and particle size determined by QELS for genipin–chitosan nanogels at pH 4.0 as function of crosslinker content.

the mixing, elastic and ionic contributions (Flory & Rehner, 1943). In the case of the genipin–chitosan nanogels at pH 4, an increase in crosslinking has opposite effects. On the one hand leads to the diminishing of ionic contribution due to the decrease of ionizable groups in the network, and to the enhancement of the elastic force. Both of these effects tend to limit the swelling of the gel. However, as crosslinker concentration increases (up to 7.5 mol.%), the mixing contribution is drastically reduced, due to the hydrophobic and closed nature of the network, and aggregation takes place, increasing the measured hydrodynamic diameter. This may explain that the determined particle sizes of the nanoparticles varying the content of genipin were fairly similar each other (Fig. 6).

At constant degree of crosslinking, when the external pH was varied it was found that the nanoparticles swell slightly at pH below ~5.5 (Fig. 7). This may be due to the protonation of free amine groups of chitosan chains at low pH, which results in an electrostatic repulsion charge favouring the penetration of water into the gel. For basic pHs the precipitation of the nanogels takes place and particle size or could not be measured or increased as consequences of particle aggregation. Studies of the pH-responsive swelling of genipin–chitosan macrogels also did not displayed an abrupt phase transition (Kaminski, Zazakowny, Szczubiałka, & Nowakowska, 2008) that was observed in other studied chitosan networks (Arteche Pujana et al., 2012). In this sense, Jahren, Butler, Adams, and Cameron (2010) found that the pH-responsive phase transition decreased with crosslinking in genipin–chitosan

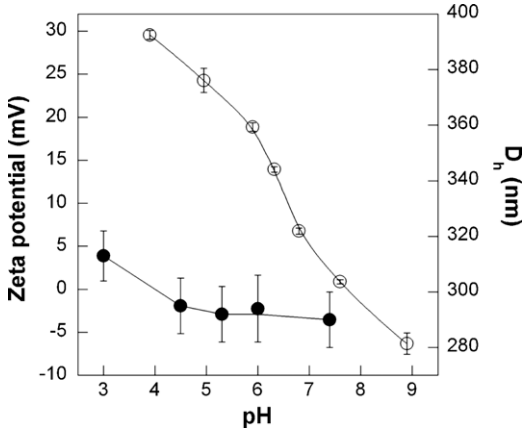

Fig. 7. (●) Average hydrodynamic diameter and (○) zeta potential of nanogels as function of external pH (for 12.5 mol.% crosslinker).

hydrogels and disappeared completely for a genipin percentage of 80 mol.%.

To obtain information about the electrical state of the ionizable groups responsible of the pH-responsive behaviour of the nanoparticles, zeta potential of genipin–chitosan nanogels was measured as function of genipin content (Fig. 6), as well as, of pH (Fig. 7). In all samples the value of the zeta potential increased up to 20 mV when the pH increased from 4 to 9. All nanogels showed positive charge at acidic conditions, so all of them maintained the chitosan mucoadhesive and absorption enhancement potential properties.

It is shown in Fig. 6 that the zeta potential values of protonated networks (pH 4.0) decreased as the nanogels crosslinking was higher, proving the loss of free amine moiety along the chitosan backbone within the modification with genipin. Regarding pH (Fig. 7), as the chitosan chains contain free amine pH-ionizable groups, a pH variation modifies the electrical state of the network. The amine primaries groups are protonated in acidic media, and the surface charge is positive and increases along the ionization process occurs. So, the progressive protonation of amino groups as pH decreases was confirmed by measuring the electrokinetic potential of the nanogels.

4. Conclusions

Chitosan low-size nanogels were prepared by the combination of two well-known methods such as, reverse microemulsion and crosslinking by genipin. Nanogels sizes ranging from 3 to 20 nm determined by TEM, and 270 to 390 nm determined by QELS in aqueous media, could be obtained by this covalent crosslinking method. Loosely crosslinked nanogels displayed an improved water solubility compared with pure chitosan. The variation of water solubility of chitosan due to the crosslinking with genipin could be consider as a compromise between the decrease of crystallinity and the elastic force within the generated network. This elastic contribution may play an essential role in the water solubility and pH-sensitive swelling of the nanogels due to the low molecular weight and rigid nature of genipin molecule.

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