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"Water dispersible pH-responsive chitosan nanogels modified with biocompatible crosslinking-agents"

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ABSTRACT

Chitosan nanoparticles were obtained by crosslinking with two biocompatible dicarboxylic acids: polyethylene glycol dicarboxylic acid and tartaric acid. The water-in-oil (W/O) microemulsion method yielded particle sizes around 10–15 nm in the dried state (TEM) and 200–700 nm in the swollen state (QELS) from commercial chitosan. All the synthesized nanogels showed improved water solubility and most of them were stable at physiological pH. QELS studies revealed the influence of the hydrophilic character and flexibility of the crosslinker on the swelling behaviour of the nanogels. The nanoparticles showed a pH-sensitive volume transition that was consistent with the p*Ka* of chitosan. The collected zeta potential data corroborated the electrostatic repulsion mechanism responsible for the pH-responsive behaviour.

1. Introduction

The well known biological and physicochemical properties of chitosan lead to the identification of this biopolymer as one of the most promising biomaterials. Chitosan, poly[β-(1-4)-linked-2-amino-2-deoxy-D-glucose], is a natural, linear and cationic polyaminosaccharide obtained by alkaline partial deacetylation of chitin. This polymer can be degraded by general lysozymes in the body and subsequently excreted as non-toxic, nonimmunogenic, and noncarcinogenic degradation products [1,2].

In addition to biodegradability, chitosan exhibits many valuable characteristics such as low toxicity and biocompatibility. For these exceptional features, as well as for being an inexpensive material, chitosan has attracted interest in various fields including the food industry, cosmetics, water treatment, agriculture and more recently in biomedicine. Researches in the latter field have shown that chitosan exhibits many interesting properties such as, immune stimulating properties [3] suppressing tumor growth [4], promoting resistance to infections by microorganisms [5], and enhancing both humoral and cell-mediated immune responses [6].

Moreover, in contrast to many other natural polymers, chitosan has a unique cationic nature [7] and is mucoadhesive [8]. Mucoadhesive properties are due to molecular attractive forces formed by electrostatic interaction between positively charged chitosan and negatively charged mucosal surfaces. Thus, it can be used to enhance drug penetration across the mucosa. Consequently, in recent years chitosan has also been extensively investigated as a carrier for mucosal drug delivery [9], as an absorption enhancer [10], as suitable material in ophthalmology and as carrier for gene delivery [11].

However, despite all its favorable features, the use of chitosan in biomedical field is limited by its poor solubility in physiological media [2]. The development of water soluble chitosan is a prerequisite for a successful biomedical use of chitosan and its derivatives. The water solubility of chitosan has been improved by different methods such as depolymerization [12], quaternization of the amino group [13], *N*-carboxymethylation [14], and PEGylation [15].

The presence of reactive hydroxyl and amino groups enhances the easy modification of chitosan to create new biofunctional materials. Reactions involving the $-NH_2$ groups at the C-2 position are the most frequently employed [16]. In particular, the possibility to modify chitosan by crosslinking has attracted attention as a new and exciting way to develop sophisticated biocompatible and biodegradable gels [17]. Chitosan gels have been prepared as macroscopic hydrogels [18], films [19], microspheres [20] or nanoparticles [21].

Chitosan nanoparticles are typically obtained by physical or covalent crosslinking. Physical crosslinking is based on ionic gelation method. Sodium tripolyphosphate [22] is the most employed ionic crosslinker. However, the produced particles are not water

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dispersible at physiological pH. Since these particles are formed by electrostatic interactions, changes in the pH of the medium could disrupt its stability [23,24]. Consequently, covalent crosslinking is more interesting than the ionic gelation method. In covalent crosslinking reaction, crosslinkers are molecules with at least two reactive functional groups that allow the formation of bridges between chitosan chains. The most common crosslinkers of chitosan are aldehydes [25], epoxides [26], and other agents [27,28]. Some of these crosslinkers, such as glutaraldehyde, are toxic [29]. Thus, using non-toxic alternative crosslinker is desirable to prepare chitosan crosslinked networks for biomedical uses.

Poly(ethylene glycol) (PEG) is one of the most popular polymers for the chemical modification of biomaterials. PEG shows exceptional physicochemical and biological properties, such as high solubility in water, biocompatibility, and ease of chemical modification. Thus, PEG has been extensively used for surface modification of chitosan improving the solubility of the modified polymers [30].

Several studies have been published on chitosan covalently crosslinked with PEG [31,32]. Bodnar et al. [33] prepared crosslinked chitosan nanoparticles by the direct amidation reaction of the activated carboxylic groups of PEGdiacid and amino groups of chitosan. These authors also employed natural di- and tricarboxylic acids with short chains, including tartaric acid, for the intramolecular crosslinking of chitosan [34]. These systems were stable in aqueous medium at pH 6.5. However, no studies were performed at physiological pH. In these investigations the particle size measured by TEM was ranged from 60 to 280 nm [34]. The particle size was reduced to 4–24 nm by previous degradation of the precursor chitosan [33].

On the other hand, reverse (water-in-oil, W/O) microemulsion has emerged as an effective way to prepare chitosan covalently crosslinked nanoparticles. Microemulsion is a transparent, isotropic and thermodynamically stable medium [35]. Colloidal aqueous droplets of chitosan acidic solution plus crosslinker are dispersed in a continuous oil phase and stabilized by surfactant molecules at the water/oil interface [36]. These colloidal sized droplets act as nanoreactors where the shape and size distribution of particles are controlled. Several works have focused on covalently crosslinked chitosan prepared by this method. However, most of these researches have employed glutaraldehyde as crosslinker agent [37,38]. Tallury et al. [39] reported a preliminary study of the synthesis of chitosan nanoparticles crosslinked with tartaric acid by water-in-oil (W/O) microemulsion instead of the direct crosslinking reaction in water. This method led to highly monodispersed nanogels with a particle size of 26 nm in the dried state. Tartaric acid–chitosan nanogels prepared by microemulsion showed high water solubility. Nevertheless, the effect of the external pH was not considered in the investigation. Instead of microemulsion medium more complex systems based on block copolymers have been developed as nanoreactors for chitosan crosslinking in order to avoid organic solvents [40].

Another exploited property of chitosan is its weak base nature. Its pKa is 6.3–7 [41]; depending on its molecular weight and deacetylation degree. The glucosamine units are protonated at pH ~ 6.5 and chitosan behaves as a soluble cationic polyelectrolyte in dilute acidic solutions. This fact is the origin of the pH-responsive swelling behaviour of covalently crosslinked chitosan. At acidic pH the accumulation of opposite electrostatic charges leads to the electrostatic repulsive force that causes the swelling of the network.

The purpose of this paper is to gain deeper insight into the swelling behaviour of chitosan nanoparticles prepared via reverse microemulsion method by crosslinking with PEGdiacid and tartaric acid. The nanogels were prepared by a slight modification of the procedure reported by Tallury et al. [39] The effect of the crosslinking ratio and crosslinker nature on several important physicochemical properties of the nanogels, such as, particle size, solubility, swelling properties, surface charge and pH-sensitivity are evaluated. This study revealed significant dependences that clarify and provide helpful support in the knowledge of the swelling behaviour of this promising biomaterial for biomedical applications.

2. Experimental

2.1. Materials

Aldrich low molecular weight chitosan was purified by a previously described method [42]. The deacetylation degree of chitosan measured by NMR was 79% which is in good agreement with the value reported by the supplier (75–85%). The viscosity average molecular weight of chitosan measured by an Ubbelohde capillary viscometer (HAc 0.3 M/NaAc 0.2 M, 25 °C) [43] was 66,000 g/mol. Poly(ethylene glycol)bis(carboxymethyl)ether, $M_n = 600$, (PEGdiacid), tartaric acid, triton X-100, *N*-hydroxysuccinimide (NHS), 1-ethyl-3-(3-dimethylaminopropyl carbodiimide hydrochloride) (EDC), Folic acid 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.

2.2. Synthesis of nanoparticles

The crosslinked nanoparticles were obtained by mixing separately prepared chitosan and crosslinker agent microemulsions. First, a chitosan solution was prepared by dissolving 1.0 g of chitosan powder in 100 ml of 1% acetic acid. Cyclohexane, *n*-hexanol and chitosan solution were mixed in a flask in a fixed ratio of 2.75:1:1 (v/v). The chitosan microemulsion was formed by adding Triton X-100 drop by drop into the mixture under vigorous stirring until the mixture became transparent. The W/O microemulsion of crosslinker (PEGdiacid or tartaric acid) was prepared following the same procedure but the diacid was previously activated with *N*-(3-Dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (EDC) and *N*-hydroxysuccinimide (NHS). In order to ensure the efficient activation, the crosslinker and NHS were first mixed in water for 15 min and then EDC was gradually added. The ratio between EDC, NHS and COOH was 5:2:1 (v/v) and the pH adjusted to 5.4 by the addition of 2 M NaOH solution. The mixture was stirred at room temperature during 4 h.

The crosslinking reaction took place during 24 h at room temperature after the addition of the crosslinker microemulsion into the chitosan microemulsion. The nanoparticles were isolated and washed by dispersion in ethanol followed by centrifugation. Finally, the obtained nanogels were dispersed in acetic acid solution, ultrafiltered and dried.

2.3. Methods

2.3.1. ^1H NMR

The crosslinked chitosan samples were analyzed by NMR spectroscopy. ^{1}H NMR spectra were recorded on a Bruker Avance 500 MHz instrument. The samples were dispersed in 2% w/w CD_3COOD/D_2O.

2.3.2. FTIR

Infrared spectra of the samples were recorded on a Thermo 520 Fourier transform infrared spectrophotometer equipped with a Smart Orbit diamond ATR (attenuated total reflection) accessory.

Spectra were taken with a resolution of 4 cm^{-1} and were averaged over 64 scans.

2.3.3. QELS

To determine the size distribution of the nanogels, a QELS spectrometer was used at an angle of 90°. Intensity correlation function measurements were carried out using a Brookhaven BI-9000AT 522-chanel digital correlator equipped with a water-cooled Argon-ion laser operating at 514.5 nm as a light source. The dried powder samples were dispersed in doubly distilled water (1 mg/L) at different pH values. The obtained homogeneous dispersions were diluted until the concentration was 40 mg/L. All measurements were made at room temperature.

The average hydrodynamic diameters were calculated by the NNLS (non-negative least squares) method from a total of 20 measurements of samples from the same batch. The uncertainties represented the standard deviation of the mean of the replicate runs.

2.3.4. Zeta potential

Electrophoretic mobility measurements were performed with a Zeta-Sizer IV (Malvern Instruments) equipment. Chitosan particles were first dispersed in acid water solution (pH = 4), then samples with different pH values were prepared by the addition of 2 M NaOH solution. The final chitosan concentration was 40 mg/L. The average values and uncertainties were calculated as has been explained for QELS measurements.

2.3.5. TEM

The morphological characteristics and particle size distribution of dried nanoparticles were studied with a Philips CM120 transmission electron microscope operating at 120 kW. A drop of nanoparticles aqueous dispersion was settled on a carbon-coated TEM grid and it was dried and glow-discharged in a high vacuum chamber.

2.3.6. UV–vis

UV–vis spectroscopy was employed for the study of water solubility of the obtained nanogels. Solubility of synthesized crosslinked chitosan nanoparticles was measured at different pH values. Briefly, nanogels were dissolved in 1% HAc solution (1 mg/mL) and the pH of the solution was adjusted by the addition of 2 N NaOH solution. The transmittance of the solution at 750 nm was recorded on a UV–vis spectrophotometer (Cintra 303 UV–vis Spectrophotometer).

3. Results and discussion

3.1. Preparation and characterization of nanoparticles

Chitosan was crosslinked by reverse microemulsion method with two biocompatible dicarboxylic acids with different chain length: tartaric acid and PEGdiacid. The carboxylic groups of the crosslinkers were previously activated with EDC and NHS. A w/o microemulsion was prepared with the activated diacid without any purification as has been above described. Separately, chitosan microemulsion was formed and the amidation reaction leading to the formation of crosslinked nanogels took place as is schematically displayed in Fig. 1. Different chitosan/crosslinker ratios were used to prepare nanosized chitosan particles according to the data summarized in Table 1.

3.1.1. FTIR studies of crosslinked nanoparticles

The structure of the synthesized nanogels was analyzed by FTIR spectroscopy.

Fig. 2 shows FTIR spectra of pure chitosan and its (A) PEG–chitosan nanogels and (B) tartaric acid–chitosan nanogels with different percentages of crosslinking agent. The spectrum of pure chitosan exhibits an absorption band around 1647 cm^{-1} corresponding to the amide I mode (C=O stretching) of *N*-acetylglucosamine units. The peak at 1593 cm^{-1} corresponds to the –NH stretching of deacetylated *N*-glucosamine groups. The intensity of amide I band (1647 cm^{-1}) and amide II band (1555 cm^{-1}) increased with crosslinking. This is due to the absorption of the new carbonyl moieties formed by the reaction of chitosan $-NH_2$ groups with the activated carboxylic groups of the crosslinkers.

For samples with high content of crosslinker, the NH_2 peak at 1593 cm^{-1} disappeared, indicating the complete reaction of amino groups in chitosan, and a new shoulder at 1730 cm^{-1} appeared. This new peak could be attributed to ester bond formation between chitosan and unreacted *N*-hydroxysuccinimide units. As Fig. 2 shows, the ester band is less intense for nanogels prepared with PEGdiacid suggesting that this crosslinker is more efficient for crosslinking with chitosan than tartaric acid. The shorter length of tartaric acid molecules may restrict the number of chitosan amino groups available for the crosslinking reaction.

Although pure chitosan shows bands at 1309 cm^{-1} and 1430 cm^{-1} (C–H bending), a remarkable increase in the intensity of these bands was observed for PEG-modified systems which agrees with the larger amount of $-CH_2$ units of this crosslinker.

3.1.2. Determination of the compositions of the nanogels

The degree of crosslinking was evaluated by ^1H NMR. Fig. 3C shows a typical ^1H NMR spectrum of chitosan. The peak at 2.0–2.1 ppm is due to the three protons of *N*-acetylglucosamine (GlcNAc) units, and the peak at 3.1–3.2 ppm corresponds to H-2 proton of glucosamine (GlcN) residues which represent the free amino group content of chitosan. A clear decrease in the 3.1–3.2 ppm peak intensity was observed in all the crosslinked samples due to the formation of amide bonds between the amine groups of chitosan and the activated carboxylic groups of the crosslinking agents. Moreover, as Fig. 3A shows, the resonance signal of new formed H-2 proton of amide moiety was observed at 4.0–4.1 ppm in the case of PEG–chitosan samples.

The non-anomeric protons of chitosan, which are connected to ring-skeleton in a glycosyl residue, have similar electron densities and similar chemical shifts. In the spectrum of the linear chitosan, the signals of the non-anomeric protons overlap and produce a broad signal which is observed between 3.5 and 4 ppm. In the case of PEG–chitosan samples crosslinking was also observed by the typical broad signal of alkyl protons of $-O-CH_2-CH_2-O$ fragments of the incorporated PEG. As can be observed in Fig. 3A, this contribution overlaps with that of the non-anomeric protons of chitosan.

The ^1H NMR spectra of tartaric acid–chitosan nanogels exhibited an isolated peak at 4.5 ppm corresponding to the resonance of H_c protons of tartaric acid units, HOO–CO–CH_cOH–CH_cOH–COOH (Fig. 3B). The appearance of this resonance indicates the successful reaction of linear chitosan with tartaric acid.

The assignments and chemical shifts of the ^1H NMR signals are given as follows: chitosan ^1H-RMN (D_2O/CD_3COOD, 500 MHz, 20 °C): δ = 4.85 (1-H of GlcN), 4.75 (1-H of GlcNAc), 3.45–4 (3-H, 4-H, 5-H, 6-H, 2-H of GlcNAc), 3.21 (2-H of GlcN), 2.08 ($H_{N-COCH3}$). PEG–chitosan ^1H-RMN (D_2O/CD_3COOD): δ = 4.85 (1-H of GlcN), 4.75 (1-H of GlcNAc), 3.45–4 (3-H, 4-H, 5-H, 6-H), 3.21 (2-H of GlcN), 2.08 ($H_{N-COCH3}$), 3.5–3.75 (a-CH_2, b-CH_2 and c-CH_2 of PEGDC), 4.05 (2-H of crosslinked GlcNH). Tartaric acid–chitosan ^1H-RMN (D_2O/CD_3COOD): δ = 4.85 (1-H of GlcN), 4.75 (1-H of GlcNAc), 3.45–4 (3-H, 4-H, 5-H, 6-H), 3.21 (2-H of GlcN), 2.08 ($H_{N-COCH3}$), 2.8 (NHS), 4.45–4.55 (a'-CH_2 of tartaric acid).

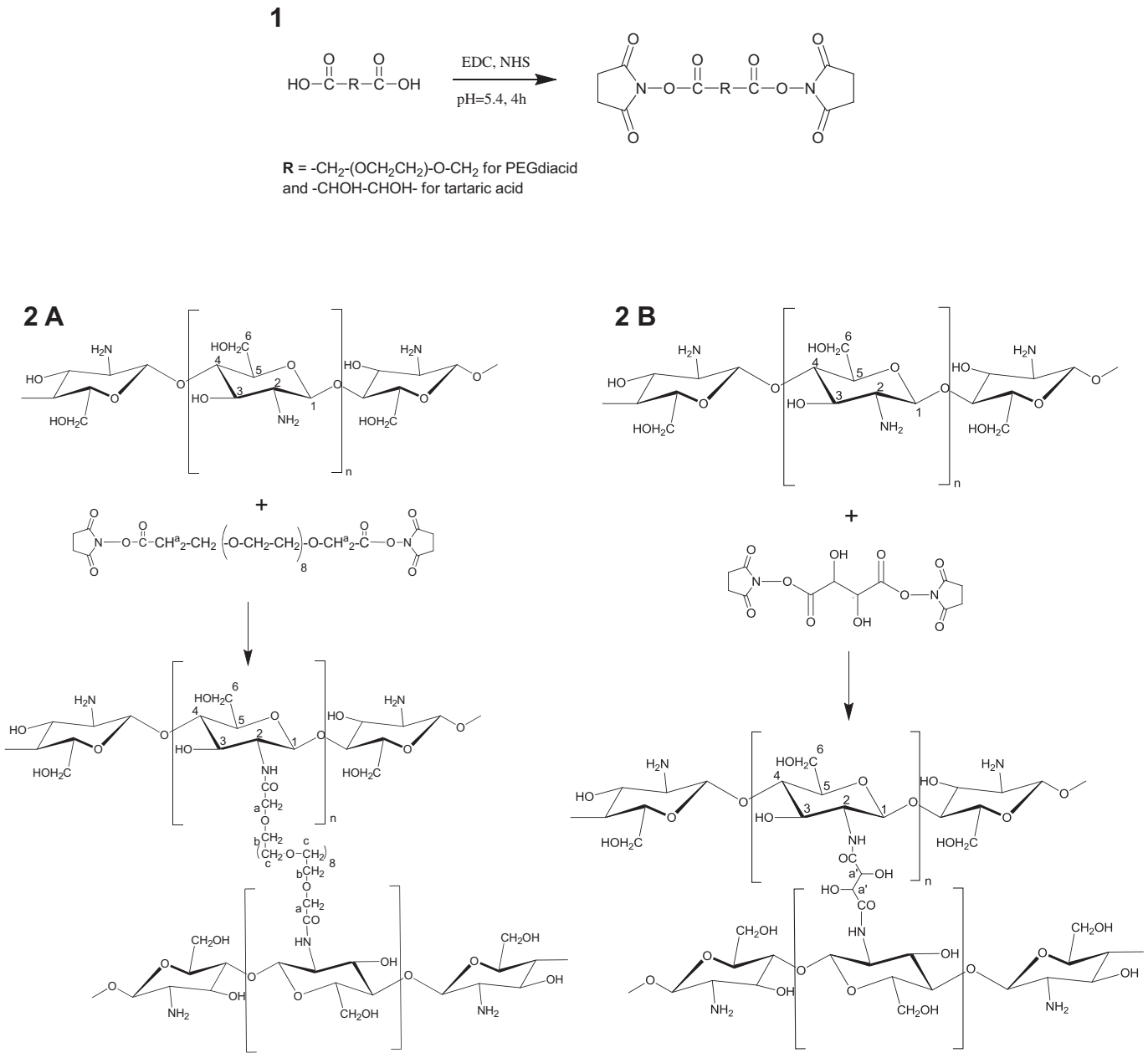

Fig. 1. Activation of the diacids (1) and crosslinking reaction of linear chitosan with PEGdiacid (2A), and tartaric acid (2B).

Table 1
Feed compositions in the preparation of crosslinked chitosan nanoparticles.

	Chitosan (mg)	PEG (mg)	Tart (mg)	EDC (mg)	NHS (mg)
Chi-PEG15.0	200	57.5	0.0	150	44.0
Chi-PEG 25.0	200	96.0	0.0	250	73.5
Chi-PEG 30.0	200	115.0	0.0	300	88.0
Chi-PEG 37.5	200	144.0	0.0	375	110.0
Chi-PEG 45.0	200	172.5	0.0	450	132.0
Chi-PEG 75.0	200	287.5	0.0	750	220.0
Chi-Htart 15.0	200	0.0	14.4	150	44.0
Chi-Htart 25.0	200	0.0	24.0	250	73.5
Chi-Htart 30.0	200	0.0	28.8	300	88.0
Chi-Htart 37.5	200	0.0	36.0	375	110.0
Chi-Htart 45.0	200	0.0	43.0	450	132.0
Chi-Htart 75.0	200	0.0	72.0	750	220.0

Among various bands of the ^{1}H NMR spectrum of chitosan, the one corresponding to the methyl protons at 2.0–2.1 ppm, possess the highest resolution [44] and was used as reference for the quantitative determination of the composition of the nanogels. This was done by analyzing the decrease of the peak located at 3.2 ppm in the ^{1}H NMR spectra assigned to H-2 protons of glucosamine moieties. The compositions of the nanogels determined by ^{1}H NMR relating to the glucosamine residues of pure chitosan are shown in Table 2. The overall degree of crosslinking of chitosan was estimated assuming that no intramolecular crosslinkings are formed ([(Total modified $-NH_2$) – (Activated –NHS pendant chains)]/2). The differences between the stoichiometric crosslinker feed and the modification rate of the chitosan reveals the typical incomplete activation of the carboxylic acid moieties [45].

Samples with incomplete crosslinking showed a signal at 2.8 ppm corresponding to the unreacted –NHS groups of the

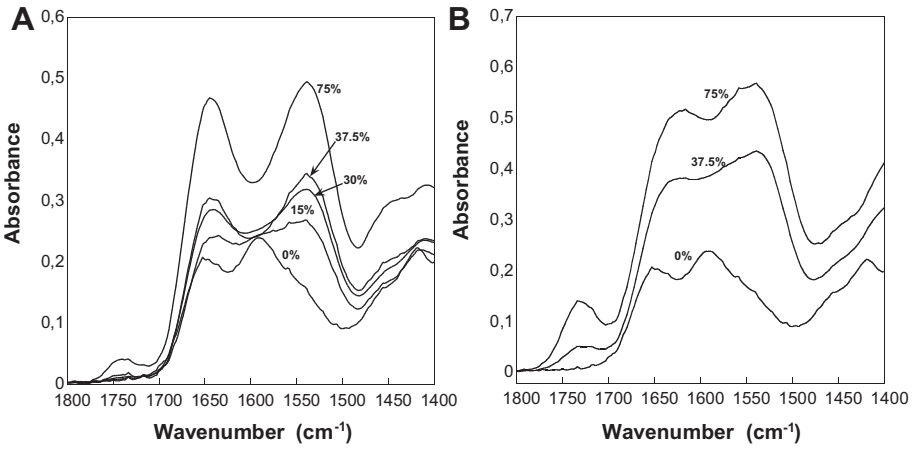

Fig. 2. FTIR spectra of chitosan nanogels modified with PEG (A) and tartaric acid (B) for different contents of crosslinker in the feed.

activated crosslinker. A typical spectrum of this incompletely crosslinked chitosan is shown in Fig. 3B. The free amine content and the overall degree of modification of the nanogels can be determined by integrating this signal. The presence of –NHS residues was observed in all the samples crosslinked with tartaric acid and only for highly crosslinked PEG nanogels, as is shown in Table 2. This fact is consistent with the FTIR analysis and again reveals the influence of the chain length of the crosslinker on the crosslinking efficiency.

3.2. Size and morphology of nanoparticles

The chitosan nanoparticles characterized by TEM showed a more or less spherical geometry, favorable solubility and no severe agglomeration of the particles. As can be seen in Fig. 4, the nanoparticles showed mean diameters ranging from 10 to 50 nm and a narrow size distribution.

The particle size of swollen nanogels was determined by quasielastic light scattering (QELS) measurements, showing a typical polydispersity index of 0.15. Fig. 5 shows a representative size distribution profile. Since chitosan is an adhesive polymer in aqueous solution, these nanoparticles tend to form aggregates. So, for interacting particles, the average particle size is always found higher than the actual size of the particles [46]. The progressive dilution reduces the inter-particle interaction and therefore, the formation of large aggregated particles. In order to reduce the presence of aggregates and, as have been proposed by other authors [47], we measured the sizes of the dispersed nanogels at highly diluted conditions.

The measured hydrodynamic diameters of the nanoparticles reveal a large swelling capacity of the gels that depends on the degree of crosslinking. The nanoparticles crosslinked with PEG-diacid showed a hydrodynamic diameter 5–35 times higher than the observed by TEM for the dried samples. In the case of the gels prepared with tartaric acid this factor varied between 9 and 20.

The swelling properties of the nanogels depend on three factors: the osmotic pressure, the electrostatic contribution and the elastic force of the network. On the one hand, the swelling is dependent on the osmotic contribution due to polymer–solvent interaction of the network. The modification with PEGdiacid or tartaric acid results in a stronger interaction between network and water increasing the osmotic factor. The swelling of polymeric networks also depends on the electrostatic factor. The free amino groups of chitosan are protonated at acidic pH values (pKa $\approx$ 6–6.5). The charge repulsions between neighbouring protonated groups cause an increase in the swelling of the nanogels. Finally, the third factor controlling the swelling degree of nanogels is the negative effect of the elastic contribution of crosslinked polymer chains which reduces the swelling capacity of nanogels as crosslinking increases [48].

By increasing the degree of crosslinking, the number of amine group decreases and this fact adversely affects the ionic contribution to the swelling. Additionally, the network becomes more compact and the swelling is limited. However, simultaneously the hydrophilicity of the system increases as a result of the increase of PEG or tartaric acid content. The introduction of pendant groups on amine primaries of chitosan leads to a decrease in the intermolecular interactions attributed to the destruction of the rigid crystalline structure of chitosan enhancing the hydrophilicity [49].

On the other hand, there are some additional factors that could affect the swelling behaviour of the nanogel but are difficult to quantify. For instance, a possible increase of chitosan content per nanoparticle for high crosslinker feeds, the contributions of intramolecular crosslinks and the presence of mono tethered crosslinker molecules.

As shown in Fig. 6 an increase in the degree of crosslinking results in a higher particle size for both systems. This trend is remarkably more pronounced for PEG-crosslinked nanogels than for the nanoparticles crosslinked with tartaric acid. In case of PEG nanogels the remarkable increase in the hydrodynamic diameters for the nanoparticles with higher crosslinker contents can be attributed to the flexibility and high hydrophilicity of this polymer ($\chi = 0.41$), probably the presence of mono tethered PEG molecules (detected in NMR spectra) also plays an important role. The lower swelling capacity of tartaric acid–chitosan nanogels may be the result of the shorter length and higher rigidity of the crosslinker, which restrict the previously mentioned contributions.

Previous studies of these systems for samples which had been directly crosslinked in aqueous medium did not find a strong dependence between swelling and stoichiometric ratio of tartaric acid or PEG [33,34]. The microemulsion method employed in this work leads to highly monodisperse nanoparticles and more homogeneous compositions that could explain the good correlation between the crosslinker content and the hydrodynamic diameters of the nanogels.

3.3. Water solubility of crosslinked chitosan

Chitosan is only soluble in aqueous acidic solutions, significantly restricting its application in biological fields. Therefore, the development of chitosan nanoparticulated systems with

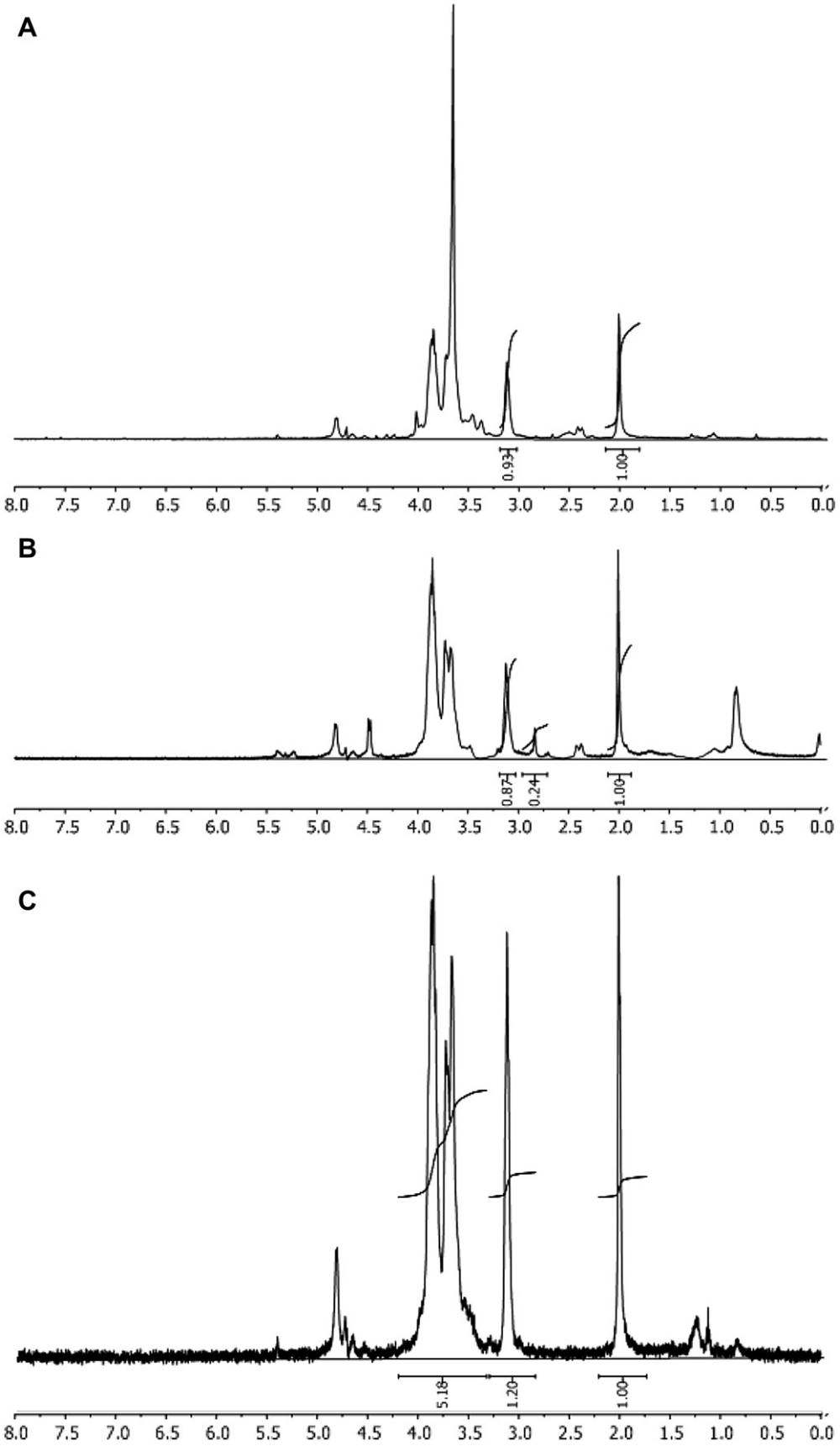

Fig. 3. ^{1}H NMR spectra of (A) PEG–chitosan nanogels (25% mol. PEG), (B) tartaric acid–chitosan nanogels (25% mol. tartaric acid) and (C) unmodified chitosan.

Table 2
Composition and overall degree of crosslinking of the chitosan nanogels determined by ^{1}H NMR.

	Stoichiometric crosslinker feed (mol. %)	$-NH_2$ modified (mol. %)	−NHS (mol. %)	Overall degree of crosslinking (mol. %)
PEG	15.0	10	0	5
	25.0	18	0	9
	30.0	18	0	9
	37.5	32	0	16
	45.0	14	0	7
	75.0	47	6	21
TART	15.0	15	4	6
	25.0	21	4	9
	30.0	26	5	11
	37.5	30	8	11
	45.0	39	9	15
	75.0	45	12	17

enhanced water solubility at physiological pH is an important goal in this research field. The water solubility of the obtained PEG–chitosan and tartaric acid–chitosan crosslinked nanoparticles was assayed as a function of pH and compared with pure chitosan.

Solutions changed from clear to opaque by increasing the pH of the aqueous medium and were colloidally stable at room temperature. Fig. 7 shows the transmittance of aqueous solutions of pure chitosan and chitosan-nanogels crosslinked with PEG and tartaric acid as a function of pH. Decreasing the pH, as the protonation of free amino groups of chitosan chains takes place, the transmittance of aqueous dispersion and so, the solubility of nanogels was improved.

The pH value when the transmittance at 750 nm reached 50%, pH_{50}, was employed as a parameter to express the water solubility of the synthesized chitosan nanogels. The pH_{50} values obtained for different chitosan nanogels are shown in Fig. 8.

Solubility of the chitosan nanoparticles is related to the hydrophilic character of the crosslinking agent and the amount of free amino groups in the chitosan chains. As Figs. 7 and 8 shows, the increase in the solubility was more remarkable for PEG–chitosan nanogels than for tartaric acid ones. The hydrophilic nature of PEG enhances chitosan solubility at physiological pH and leads to fully dispersible nanogels even at basic pH values. The solubility of the nanogels was greater as the crosslinker content increased, except for highly crosslinked tartaric acid–chitosan nanogels. In the case of tartaric acid nanogels with 75% degree of crosslinking, the increase in the crosslinker content enhanced the opalescence of the solutions. As has been previously pointed out in the study of hydrodynamic diameters of the nanogels, this result may be related to the more compact structure of the networks prepared with tartaric acid. In fact, chitosan nanoparticles crosslinked with tartaric acid at a stoichiometric ratio of 75% precipitated in aqueous media, despite its hydrophilic character.

3.4. pH-responsive swelling properties of nanogels

The swelling behaviour of chitosan nanogel particles was studied by QELS as function of the external pH. Fig. 9 shows the

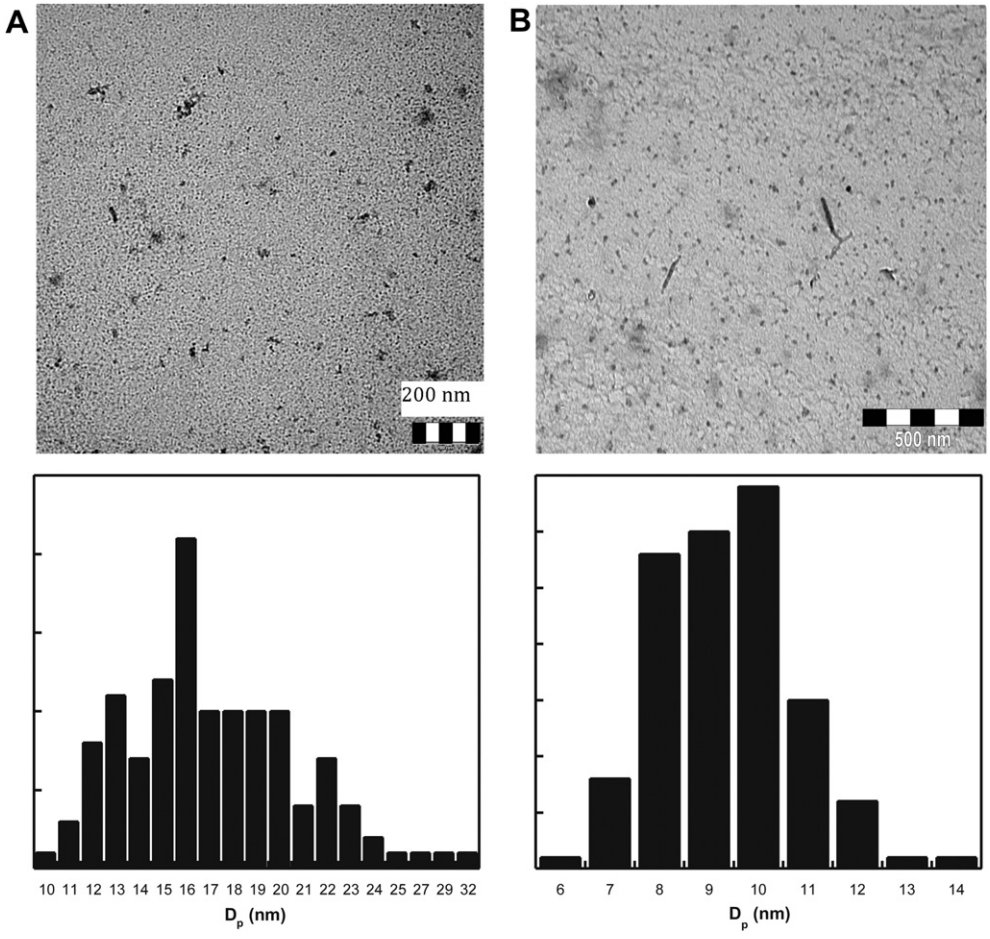

Fig. 4. TEM micrographs and particle size distribution of A) PEG 37.5% and B) tartaric acid 37.5% chitosan nanogels.

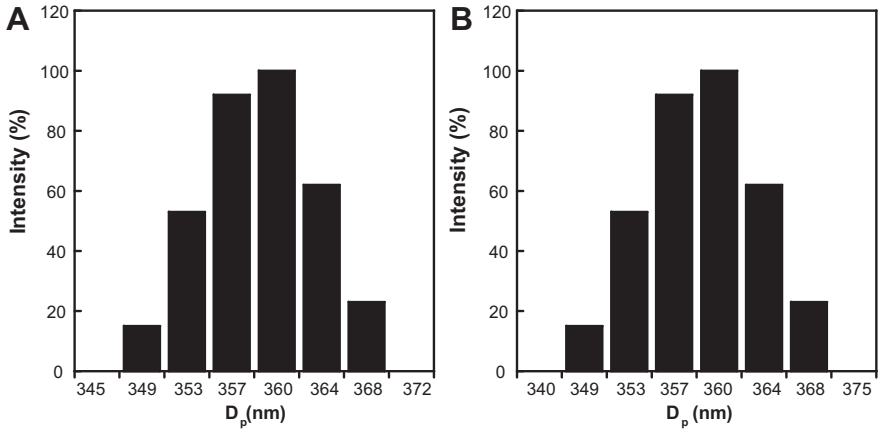

Fig. 5. QELS particle size distribution of (A) PEG–chitosan nanogels and (B) tartaric acid–chitosan nanogels with a crosslinker ratio of 37.5% at pH = 7.0.

hydrodynamic diameters of PEG–chitosan (A) and tartaric acid–chitosan (B) nanogels with the same degree of crosslinking (37.5%) measured at different pHs. It can be seen that the average size increases for both systems when the pH decreases. The primary amine groups are protonated in acidic media, and hydrodynamic diameters increase because of the repulsive interaction. Chitosan crosslinked nanogels showed a pH-responsive volume transition which was consistent with the p*Ka* value of linear chitosan chains [50]. PEG–chitosan nanogels exhibited a large volume transition (~90%) when compared to tartaric acid-modified particles (~15%), due to the more rigid network originated by this last crosslinker.

The progressive protonation of amino groups at low pH values was confirmed by measuring the electrokinetic potential (ζ-potential) of the nanogels. The value of the ζ-potential increased from (−8) − (−10) to 30–45 mV in the range of pH from 10 to 4 (Fig. 10). Although the ζ-potential characterizes the surface charge of the particles, it could be assumed that the amino groups inside the nanogel behave in a similar way to the surface ones [22].

The influence of the chemical structure of the crosslinkers was also observed in the study of the zeta potential. As Fig. 11 shows, the plot of zeta potential versus the crosslinker content showed opposite behaviours for PEG and tartaric acid.

In both systems the evolution of the zeta potential with the crosslinker content is in consonance with the observed colloidal stability of high-crosslinked nanoparticles. Thus the progressive decrease in the charge of the protonated network for tartaric acid nanogels results in the observed loss of solubility for high-crosslinked nanoparticles. The opposite behaviour in the zeta potential leads to the observed increase in the solubility of high-crosslinked PEG nanoparticles.

The different behaviour of both systems is difficult to explain if we consider that the mechanism and the degrees of crosslinking

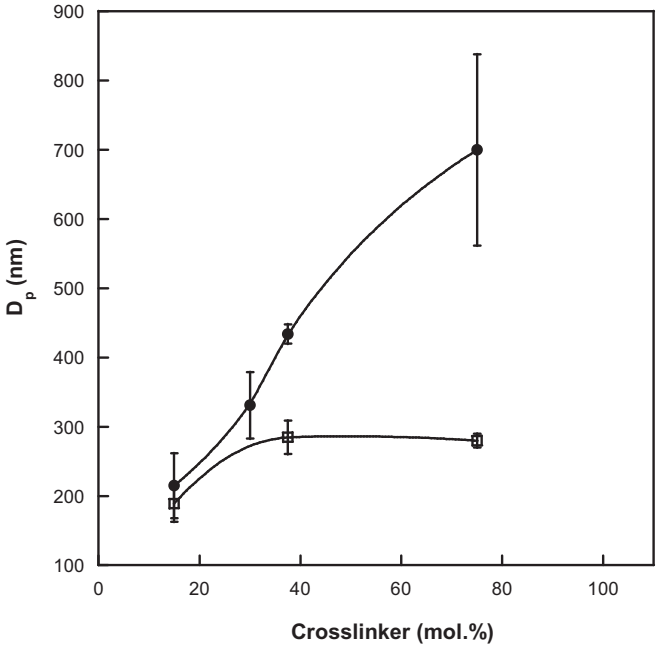

Fig. 6. Effect of crosslinker content on hydrodynamic diameter determined by QELS for (●) PEG–chitosan and (□) tartaric acid–chitosan nanogels dispersed in water at pH = 4.0.

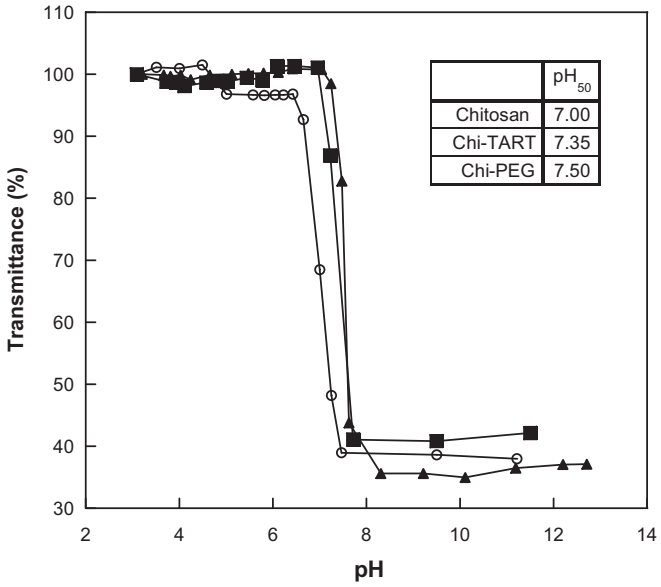

Fig. 7. Transmittance variation with external pH for pure chitosan (○), PEG–Chitosan (▲) and tartaric acid–Chitosan (■) nanogels with a modification degree of 25.0 mol%.

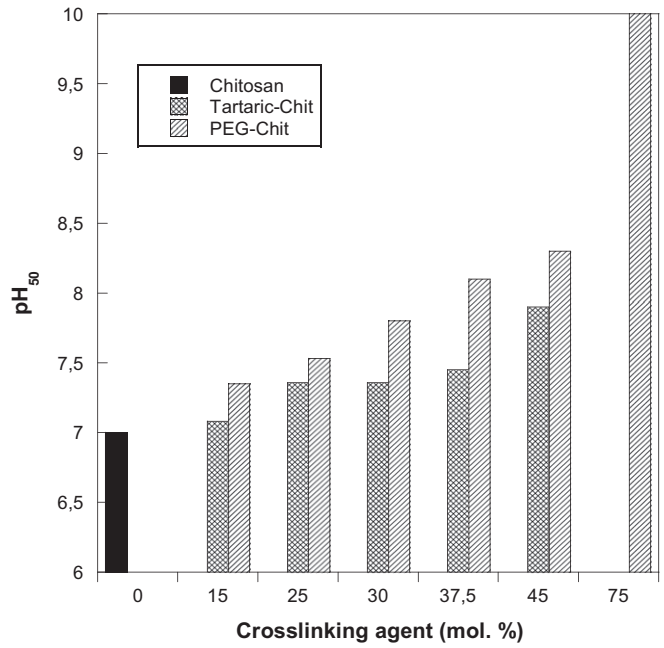

Fig. 8. pH_{50} values for pure chitosan, and dispersions of tartaric acid and PEG–chitosan nanogels.

are similar. Experimental data showed evidence that hydrolysis reaction of mono tethered crosslinker molecules had not occurred. Taking all above into consideration, the main difference between both chitosan nanoparticles is the swelling capacity (Fig. 6). Apparently, for the nanogels prepared with PEG, the progressive decrease in the charge of the protonated network resulting from the increase in the degree of crosslinking can be offset by the higher flexibility and swelling capacity of these nanogels. Thus, both factors can provide a higher net charge distribution at the particle surface. For the tartaric acid nanogels the progressive increase of the rigidity introduced by the crosslinker may acts in the opposite sense.

The p*Ka* values of synthesized nanogels were calculated from data shown in Fig. 10 using the following equation [22],

$$pK_a = pH_{\zeta=\zeta_{plateau/2}} - \frac{0.4343F\zeta_{plateau}}{2RT}$$

where, $pH_{\zeta=\zeta plateau/2}$ is the pH where the maximum value of the zeta potential "$\zeta_{plateau}$" is reduced by half. *T* is the temperature and *F*, *R*, are the Faraday and the gas constant respectively. The p*Ka* values thus obtained were 5.9 for PEG and 6.2 for tartaric acid, respectively.

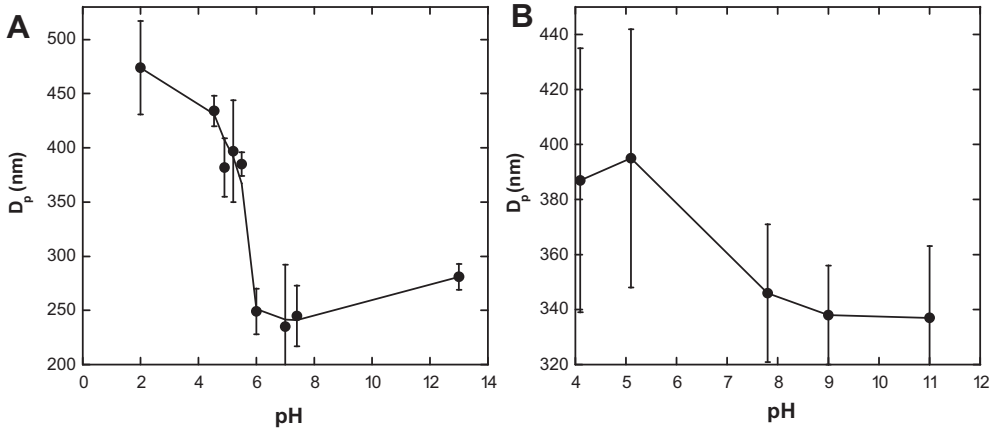

Fig. 9. Average diameter as function of external pH for (A) PEG and (B) tartaric acid–chitosan nanogels (37.5 mol% crosslinker stoichiometric ratio).

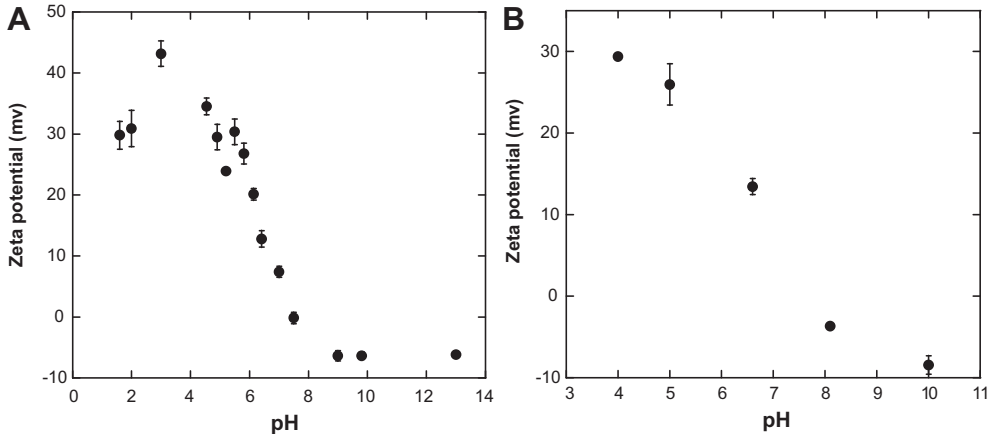

Fig. 10. Zeta potential as function of pH of the medium for (A) PEG and (B) tartaric acid-crosslinked nanogels (37.5 mol%).

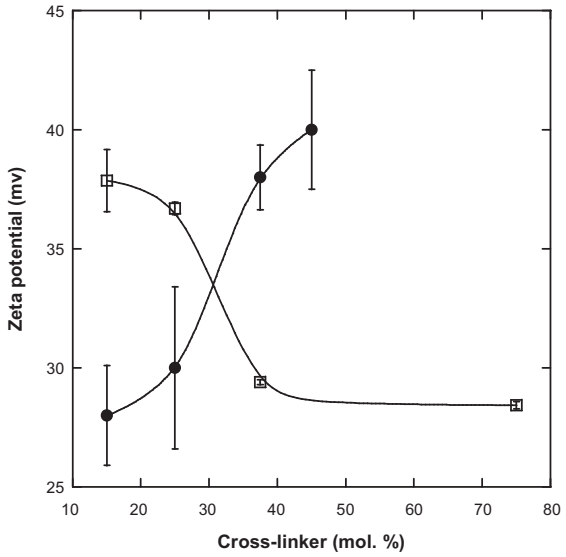

Fig. 11. Zeta potential for (□) tartaric acid–chitosan and (●) PEG–chitosan nanogels at pH = 4.0 as function of crosslinker content.

4. Conclusions

Chitosan was successfully crosslinked by amidation reaction using biocompatible dicarboxylic acids (PEGdiacid and tartaric acid) by w/o microemulsion to form ultrafine nanoparticles (10–15 nm TEM). PEGdiacid showed to be more efficient for chitosan crosslinking than tartaric acid. Water solubility of the samples was improved in comparison with linear chitosan and furthermore, in most of cases the new systems were stable at physiological pH.

The synthesized nanogels showed a pH-responsive swelling behaviour that corresponds to the p*Ka* of the non-crosslinked chitosan. The particle size of colloid dispersion varied from 250 to 450 nm by decreasing the pH from 8 to 4. The p*Ka* values of resulting nanoparticles estimated by Zeta potential measurements were around 5.9–6.2. The swelling capacity and surface charge clearly depends on the hydrophilic character and flexibility of the crosslinker.

The water solubility at physiological pH and the pH-responsive swelling of the synthesized nanogels make them attractive candidates as biocarriers for a large variety of biomedical applications.

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