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Cavitation effect on chitosan nanoparticle size: A possible approach to protect drugs from ultrasonic stress

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ABSTRACT

The objective of this study was to verify the influence of different modes of ultrasonic radiation on both the mean diameter and the polydispersity index (PI) of chitosan (CH) nanoparticles, which were prepared by means of the ionotropic gelation method. The variations in duration, intensity and mode of cycle of ultrasonic radiation allowed us to highlight several optimal treatments in order to obtain a potential carrier for site-specific drug delivery. Despite the high utility, ultrasound may be a risk factor for sensitive drug-loaded nanoparticles; in order to protect the drug from thermal or mechanical stress, the effects of ultrasonic radiation only on the CH dispersion (instead of the chitosan/tripolyphosphate (TPP) mixture) were studied, without damaging the drug added to the TPP solution. The increase of the wave amplitude, mode of cycle and time of sonication decreased the particle mean diameter; moreover, the mode of cycle showed a greater effect than the other parameters on the PI of the nanoparticle system. Both the mean diameter and the PI of CH nanoparticles increased with increasing CH concentration. The application of ultrasound only on the CH dispersion showed interesting results, particularly in regard to formulations prepared from low and medium molecular weight chitosan.

1. Introduction

Chitosan (CH) is a non-toxic biodegradable polycationic polymer with low immunogenicity. It has been extensively investigated for formulation as a carrier and delivery system for therapeutic macromolecules (Janes, Calvo, & Alonso, 2001; Kumar, Muzzarelli, Muzzarelli, Sashiwa, & Domb, 2004; Richardson, Kolbe, & Duncan, 1999).

CH nanoparticles have attracted great attention in pharmaceutical applications, including colon-targeted drug delivery, mucosal delivery, cancer therapy, vaccine delivery, and gene delivery. Since the primary amine groups carry a positive charge and mucoadhesive properties, chitosan is very useful in drug delivery applications (Agnihotri, Mallikarjuna, & Aminabhavi, 2004; Jonassen, Kjøniksen, & Hiorth, 2012; Sarmento, Ferreira, Veiga, & Ribeirov, 2006). CH nanoparticles are a very efficient and non-toxic absorption enhancer for both orally and nasally administered peptide drugs (van der Lubben, Verhoef, Borchard, & Junginger, 2001).

The ionotropic gelation method is commonly used to prepare CH nanoparticles. In acidic solution, the $-NH_2$ of the chitosan molecule is protonized to $-NH_3^+$. It interacts with an anion, such as tripolyphosphate (TPP), by ionic interaction to form a microgel particle (Bernkop-Schnürch & Dünnhaupt, 2012; Lee, Mi, Shen, & Shyu, 2001).

In addition, reversible physical crosslinking by electrostatic interaction, instead of chemical crosslinking, is applied to prevent possible toxicity of reagents and other undesirable effects (Shu & Zhu, 2000).

The CH/TPP nanosystem shows some attractive characteristics that make it a promising carrier for the delivery of macromolecules. These features include a preparation procedure under mild conditions, homogeneous and adjustable size, a positive surface charge that can be easily modulated, and a great capacity for the association of peptides, proteins, oligonucleotides and plasmids (van der Lubben et al., 2001).

Ultrasonication is a common tool for the preparation and processing of polymer nanoparticles. It is particularly effective in breaking up aggregates and reducing the size and polydispersity of nanoparticles (Grieser, Ashokkumar, & Sostaric, 1999).

It is generally agreed that ultrasonication causes main chain scissions at the 1,4-glycosidic bond without affecting the deacetylation (DD) of CH samples (Chen, Chang, & Shyur, 1997). The process has, therefore, been conveniently applied to produce CH samples of lower molecular weights of the same DD (Signini, Desbrières, & Campana Filho, 2000).

Despite the high utility of ultrasound, it may be a risk factor for sensitive drug-loaded nanoparticles; Tang, Huang, and Lim (2003)

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reported that it is not advisable to use high-intensity ultrasonication to prepare or process chitosan nanoparticles for drug delivery.

In our study, the CH nanoparticles were produced by the ionotropic gelation method and we verified the influences of duration, intensity and mode of cycle of ultrasonic treatment on the dimension and homogeneity of the nanoparticle system. Moreover, we studied the effects of both CH concentration and molecular weight on nanoparticle size.

Furthermore, to overcome the disadvantage presented by sonication, we studied the effect of ultrasonic cavitation only on the CH dispersion. This approach sought to optimize the dimension and morphology of the nano-carrier, without affecting the drug.

2. Experimental

2.1. Materials

Materials used were low, medium, and high molecular weight CH (Aldrich, USA), pentasodium TPP (Sigma–Aldrich, USA), acetic acid (Fluka, Chemie GmbH, Switzerland), and tungstophosphoric acid (Merck, Germany).

2.2. Preparation of CH nanoparticles

CH–TPP nanoparticles were prepared by the ionotropic gelation method (López-León, Carvalho, Seijo, Ortega-Vinuesa, & Bastos-González, 2005; Tang et al., 2003). Different concentration CH solutions (1, 2, 4, and 10 mg/ml) with 1% (w/w) acetic acid were prepared. TPP solution (0.84 mg/ml) was then added into the CH dispersion at a volume ratio of 5:2 (v/v) (CH:TPP). The cavitation effect exerted by ultrasonic radiation was evaluated (Bandelin, Sonoplus Ultrasonic Homogenizers HD 2070, Berlin). The reaction vessel, with a capacity of 50 ml (diameter: 4 cm), allowed the visualization of the ultrasonic probe and its immersion level, in order to position the latter always in the same position; with a reaction mixture of 21 ml (equal to a level of 1.8 cm in the vessel) the tip of the probe distanced 1 cm from the bottom of the reaction vessel.

The mean diameter and zeta potential were determined by light scattering 24 h after ultrasonic treatment.

2.3. Processing by ultrasonication

For sonication treatments, the same type of 50-ml beaker was used as for the previous treatment; thus, for each treatment, the immersion probe was placed in the same position. When evaluating sonication effects, the repeatability of the working conditions, including volume recipient, volume dispersion, and probe position, are very important because a variation of these parameters leads to different results in terms of nanoparticle size reduction and polydispersity. The instrument allowed the modification of sonication time, mode of cycle and wave amplitude. The mode of cycle is mainly distinct in being continuous or discontinuous. In the discontinuous mode (from cycle 1 to 9), the ultrasonic radiation is interrupted by breaks. In cycle 1, the time fraction of ultrasonic radiation is 0.1 s and the break is 0.9 s. As the cycles progress, the fraction of ultrasound increases and the break decreases, and, ultimately, cycle 10 consists of continuous ultrasonic radiation.

The ultrasonic radiation was exerted at an input power of 70 W. In order to evaluate the effect of each ultrasonic parameter on the nanoparticles system, sonication time, intensity and mode of cycle were modified individually. Specifically, sonication times were 1, 2, 4 and 8 min; wave amplitudes were 10, 25 and 50%; and modes of cycle were 1, 4, 7 and continuous. These parameters were combined to apply a total of 48 treatments.

2.4. Mean diameter, zeta potential and polydispersity index determination of nanoparticles

The light scattering method was used to measure the mean diameter and polydispersity index (PI) of CH–TPP nanoparticles. The scattered light intensity of the solution at 173° was measured by a light scattering photometer (ZEN3600 Zetasizer, Malvern Instruments, England).

The scattered light intensity was converted to the diffusion coefficient (D, m^2/s). The size of the nanoparticle was calculated from the diffusion coefficient of the particles using the Stoke–Einstein equation. PCS software was used for analysis (Banerjee, Mitra, Singh, Sharma, & Maitra, 2002).

The polydispersity index was used as a measure of the width of the size distribution. A PI less than 0.4 indicates a homogenous and monodisperse population.

Zeta potential was measured as the particle electrophoretic mobility by means of laser microelectrophoresis in a thermostated cell.

Every sample measurement was repeated three times and the results are expressed as mean ± standard deviation.

2.5. Isolation of particles

Nanoparticles were collected by ultracentrifugation at 40,000 rpm for 30 min. Nanoparticles were stored at 4 °C for further experiments or lyophilized.

2.6. Yield value

The production yield of the nanoparticles was obtained by ultracentrifugation of fixed volumes of the nanoparticle suspensions. The supernatants were discarded and the centrifugates were maintained at 50 °C until constant weight. The production yield was calculated by comparing the actual weight with the theoretical weight of the nanoparticles.

2.7. Fourier transform infrared (FTIR) analysis

FTIR spectra were measured using a Bruker Vector 22 spectrophotometer. Samples were lyophilized, gently mixed with micronized KBr powder and compressed into discs. For each spectrum, a 256-scan interferogram was collected with a 4 cm^{-1} resolution in the mid-IR region at room temperature.

2.8. Transmission electronic microscopy (TEM)

The morphology of CH nanoparticles was observed in a TEM (JEOL, 200CX, Japan) after staining with 1% of tungstophosphoric acid on Formvar (Cu/C) grids. Samples were observed 24 h after ultrasonication.

3. Results and discussion

3.1. Effects of wave amplitude, mode of cycle and sonication time

All three parameters (wave amplitude, mode of cycle and sonication time) influenced the size of the nanoparticles. The graph in Fig. 1a shows the dimensional values of samples obtained after the application of 12 treatments with a combination of 4 durations (1, 2, 4 and 8 min) and 3 wave amplitudes (10, 25 and 50%) with a continuous cycle mode. The results showed that the increase of the wave amplitude, at constant cycle and sonication time, decreased the particle mean diameter. In Fig. 1a, the point-lines show the decrease of the mean diameter values for increased wave amplitude and time of sonication.

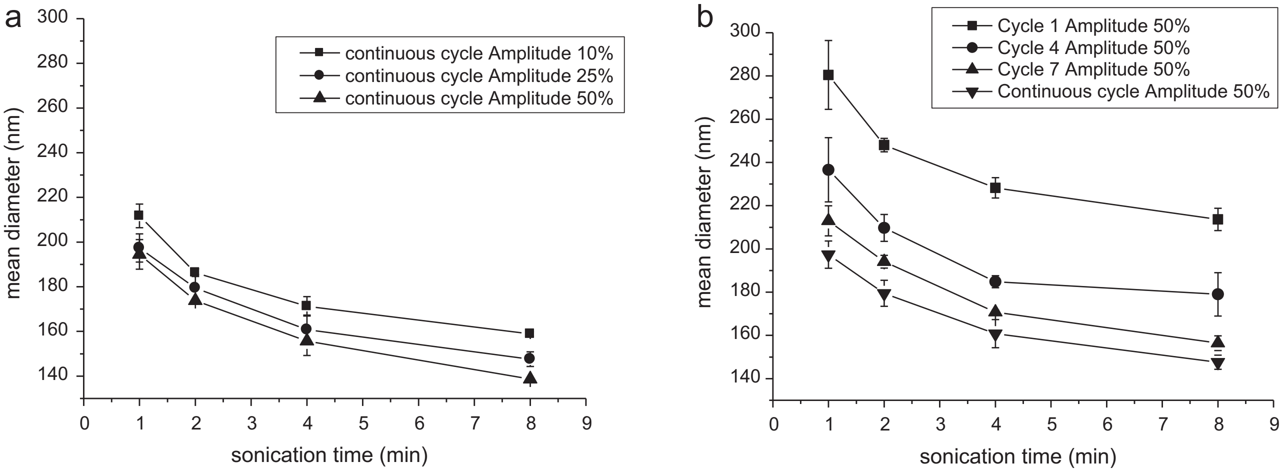

Fig. 1. (a) Mean diameter of CH nanoparticles as a function of sonication time associated with a change of wave amplitude; (b) mean diameter of CH nanoparticles as a function of sonication time associated with change of cycle modality.

The graph in Fig. 1b shows the results of 16 samples obtained from a combination of 4 durations (1, 2, 4 and 8 min) with 4 different cycles (1, 4, 7 and continuous) combined with a wave amplitude of 50%. The results showed that the increase in cycle mode, at constant amplitude and sonication time, decreased the particle mean diameter. In Fig. 1b, the point-lines show the decrease of the mean diameter values for increased cycle mode and time of sonication.

Based on the results shown in Fig. 1a and b the influence of time of sonication on nanoparticle size is quite clear. Apart from cycle mode or wave amplitude, the increase of duration of treatment resulted in a decrease of nanoparticle size. A summary of the

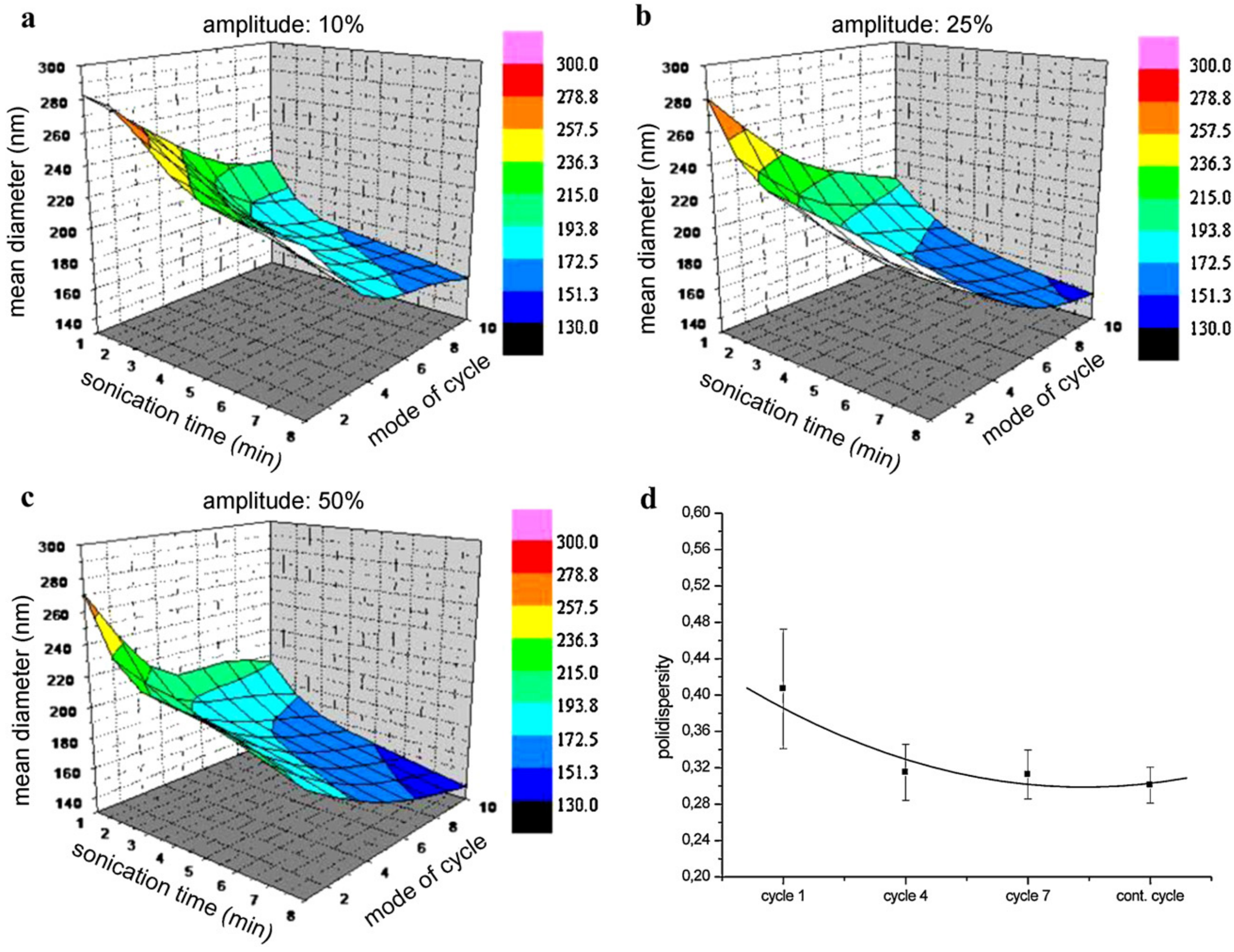

Fig. 2. (a–c) 3D diagrams show the influence of time and mode of cycle on mean size of nanoparticles. Treatments were grouped by wave amplitude of 10% (a), 25% (b) and 50% (c). (d) Polydispersity of chitosan nanoparticles as a function of ultrasonic cycle.

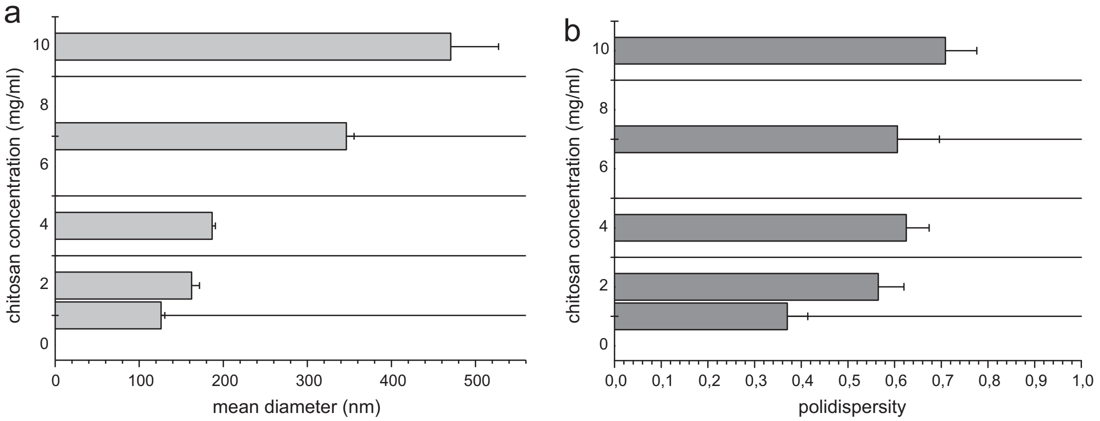

Fig. 3. (a) Size of chitosan–TPP nanoparticles. Mean diameter increased with increasing chitosan concentration. (b) The polydispersity of chitosan–TPP nanoparticles. The PI increased with increasing chitosan concentration.

effects of ultrasonic parameters can be observed on the 3D graphs presented in Fig. 2a–c, which show the influence of time and mode of cycle on mean size of nanoparticles, grouped by amplitude value.

A reduction of approximately 55% in mean diameter, compared to the control, was obtained after treatment with the highest values of duration, wave amplitude and mode of cycle.

The effect of ultrasound treatments on the polydispersity of nanoparticles was also studied. The ultrasonic parameter with the greatest effect on PI was the mode of cycle. Fig. 2d shows a leveling off of the polydispersity value at high cycle mode.

3.2. *Effect of CH concentration*

The influence of CH concentration on average size of nanoparticles was observed. Low molecular weight CH was used and five dispersions in acetic acid 1% v/v with CH concentrations of 1, 2, 4, 7 and 10 mg/ml were prepared.

The ultrasonic treatment was the same for all the samples: continuous cycle, 8-min duration and a wave amplitude of 50%. The light scattering analysis was performed on samples diluted to 1:5 with phosphate buffered saline. The results obtained are summarized in the graphs in Fig. 3.

The average size of the nanoparticles remained under 200 nm up to a CH concentration of 4 mg/ml, while the homogeneity changed significantly at CH concentrations greater than 2 mg/ml. These results confirmed that low concentrations, up to 2 mg/ml, are most suitable for drug carrier preparations.

3.3. *Effect of ultrasound application on CH dispersion*

To avoid the ultrasonic stress of the loaded drug, the influence of sonication applied only on the CH dispersion was studied.

As reported in several studies of CH/TPP nanoparticles, the drug is usually added to the TPP solution. This practice facilitated the application of ultrasound on the CH dispersion, which is the previous stage in the formation of the mixture.

Fig. 4 shows that the average size of the nanoparticles was almost the same for low, medium, and high molecular weight CH after the treatment with the highest ultrasonic parameters for the CH/TPP mixture was applied. After applying the same ultrasonic treatment only to the CH dispersion, the results showed a higher mean diameter of the nanoparticles, particularly for the high molecular weight CH dispersion.

The mean diameter of the nanoparticles obtained from the low and medium molecular weight CH showed an increase of 20–35%, compared to the sonicated CH/TPP mixture. The mean diameters of both low and medium molecular weight nanoparticles were less than 200 nm. Therefore, the results showed that ultrasound application to the CH dispersion only affected size reduction and homogenization, although to a lesser extent than application to the CH/TPP mixture.

Tsaih, Tseng, and Chen (2004), Lan, Yang, and Li (2004) and Tsai, Bai, and Chen (2008) reported that the cavitation actions take place all over the solution concurrently to degrade the CH molecule or to break up the nanoparticle cluster to fine particles. Our findings justify the dimensional variation between samples sonicated as the CH/TPP mixture and sonicated only as the CH dispersion. In fact, in the first case, sonication induced effects on both the CH molecules and the nanoparticle aggregates, which are created following the formation of the mixture. In the latter case, the sonication induced effects only on the CH molecules.

3.4. *Effect of CH molecular weight on mean diameter, zeta potential, PI and yield value (%) of nanoparticles by sonication of only the CH dispersion*

Preparations obtained from different molecular weight CH have been previously described.

Table 1 reports results of both samples that were not sonicated and samples sonicated only as the CH dispersion.

The yield values and mean diameters of sonicated low and medium molecular weight nanoparticles were quite comparable.

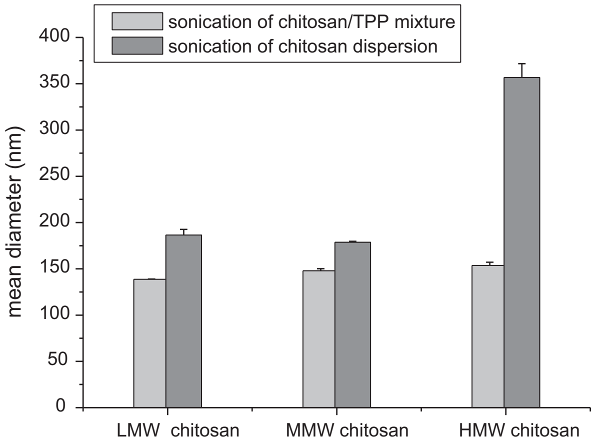

Fig. 4. Mean diameter of chitosan nanoparticles obtained by sonication of the chitosan/TPP mixture and chitosan dispersion only.

Table 1
Composition and physicochemical properties of chitosan nanoparticles.

CH HMW (%)	CH MMW (%)	CH LMW (%)	CH:TPP mass ratio	Mean diameter (nm)	PI	Zeta potential (mV)	Yield (%)
–	–	0.07	3.5:1	264 ± 2	0.48	52 ± 1.5	42.3
–	0.07	–	3.5:1	400 ± 26	0.80	57 ± 0.6	40.7
0.07	–	–	3.5:1	1234 ± 75	*n.d.*	58 ± 0.3	82.5
–	–	0.14	7:1	335 ± 31	0.48	62 ± 1.6	15.7
–	–	0.07[a]	3.5:1	186 ± 6	0.35	50 ± 1.2	42.8
–	0.07[a]	–	3.5:1	179 ± 1	0.38	50 ± 0.1	42.8
0.07[a]	–	–	3.5:1	357 ± 15	0.48	55 ± 0.3	68.7
–	–	0.14[a]	7:1	2636 ± 4	0.39	56 ± 1.1	15.7

All samples were crosslinked with 0.02% TPP solution.

[a] Sonicated only as CH dispersion samples.

The zeta potential of sonicated samples was slightly lower than non-sonicated formulations.

Sonicated low and medium molecular weight nanoparticles showed positive features in terms of average size, homogeneity and stability.

3.5. Morphological characterization of nanoparticles

Fig. 5a shows a TEM image of individual nanoparticles approximately 25 nm in diameter and a large aggregate formed with many distinctive single nanoparticles, each still possessing a similar nano-metric dimension. As shown in Fig. 5b and c, nanoparticle aggregates display polyhedral forms.

3.6. FTIR characterization of nanoparticles

Comparable FTIR spectra were obtained from non-sonicated and sonicated CH samples (Fig. 6a), which suggests the absence of chemical modifications. CH has three characterization peaks at 3260 cm^{-1}, 1092 cm^{-1} and 1650 cm^{-1}. The first peak has been attributed to —NH_2 and —OH group stretching vibration in the CH matrix. The second peak, at 1650 cm^{-1}, has been attributed to the bending of —NH_2 and the stretching of —CO of the amidic group. Moreover, near 1600 cm^{-1}, there is another peak (1570 cm^{-1}) related to —NH_3^+ bending. The third peak, at 1092 cm^{-1}, has been attributed to C—O—C stretching.

The sonicated CH showed that the peak of —OH stretching vibration shifted from 3260 to 3400–3450 cm^{-1}, which demonstrates

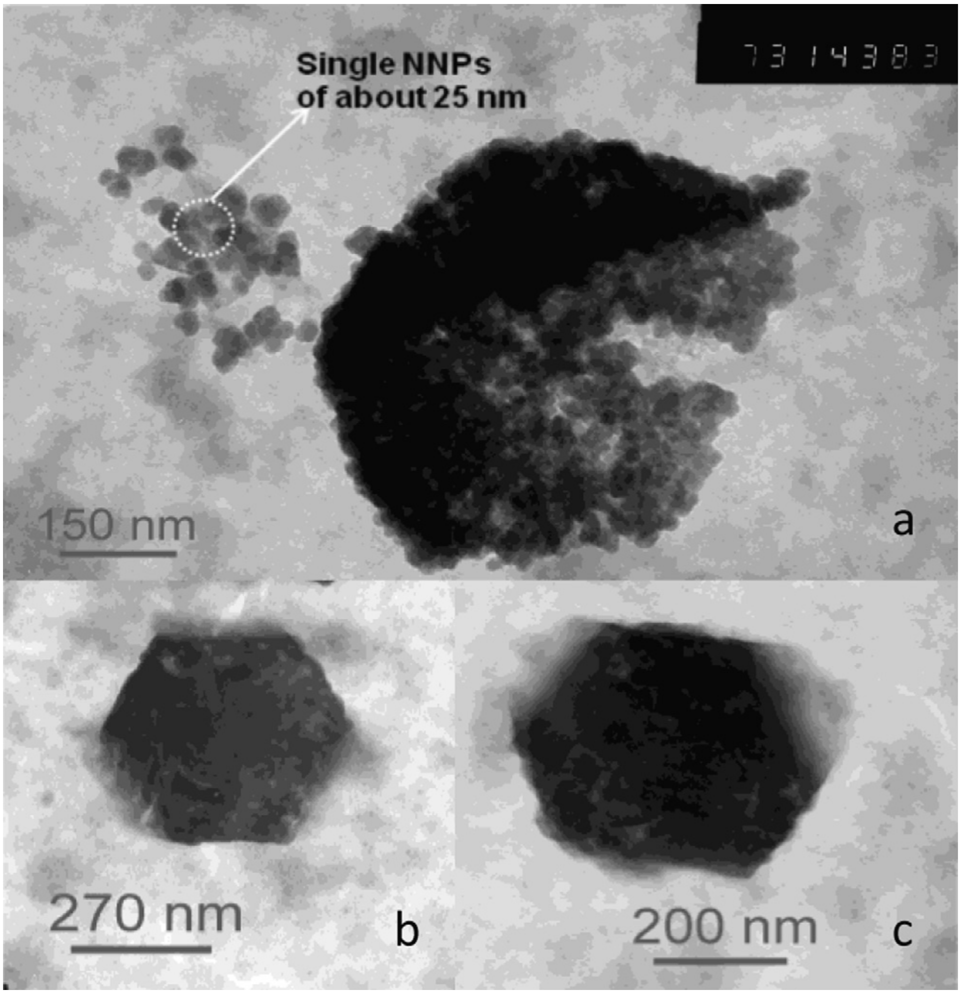

Fig. 5. TEM of sonicated chitosan–TPP nanoparticles.

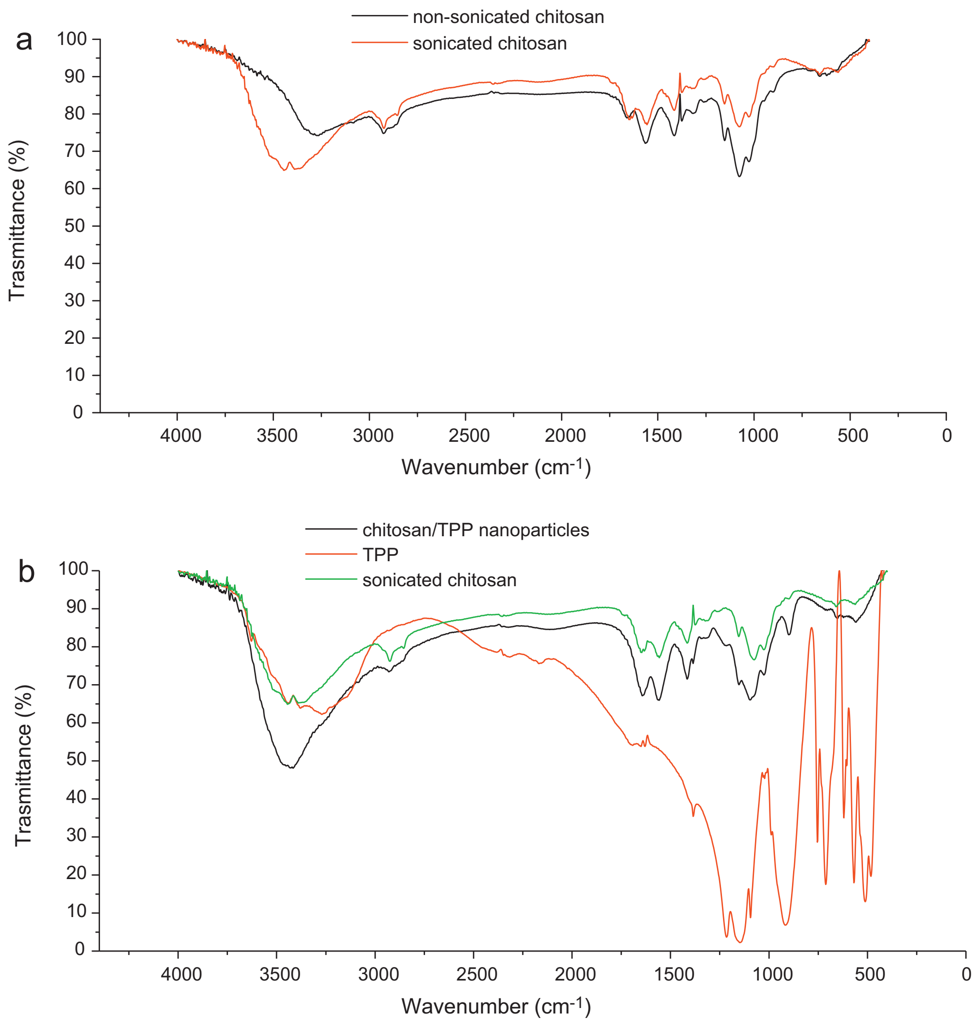

Fig. 6. (a) FTIR spectra of non-sonicated and sonicated chitosans; (b) FTIR spectra of sonicated chitosan, TPP and chitosan nanoparticles.

the probable transition from a weakly associated intramolecular —OH to a strongly associated —OH. This transition could be due to the sonication process, which, breaking CH chains, breaks the β-1,4-glycosidic bonds with subsequent formation of new hydroxyl groups and hydrogen bonds.

The FTIR spectra of the CH nanoparticles (Fig. 6b) showed, over the characteristic peaks of CH, two peaks related to the presence of sodium TPP. The first peak was at 900 cm^{-1} and attributed to P—O—P stretching and the second, at 1217 cm^{-1}, was attributed to PO stretching.

4. Conclusion

The particle size of CH–TPP nanoparticles produced by ionotropic gelation can be manipulated using ultrasonic radiation at different treatment times, modes of cycle and CH concentrations. The application of ultrasonic radiation results in different nanoparticle sizes, which may be due to different degradation mechanisms of the CH molecules and different sizes of degraded CH molecules participating in the ionotropic gelation with TPP molecules. The degradation of CH molecules by ultrasonic radiation is mainly caused by cavitation effect.

All the ultrasonic parameters showed important influences on the size of nanoparticles. The increase of the wave amplitude, mode of cycle and time of sonication decreased the mean particle diameter. Moreover, the mode of cycle showed a greater effect on the PI of the nanoparticle system than the other parameters. A reduction of approximately 55% in mean diameter, compared to the control, was obtained after treatment with the highest parameters in duration, wave amplitude and mode of cycle.

Mean diameter and PI of CH nanoparticles increased with increasing CH concentration.

The application of ultrasound only on the CH dispersion revealed interesting results, particularly in regard to formulations created from low and medium molecular weight CH, which showed, after mixture formation, a good mean diameter and PI, thus preserving the drug that had been previously added to the TPP solution.

The morphological analysis allowed us to highlight the main features of these systems, which revealed small nanoparticles and their aggregates with polyhedral shapes.

FTIR analysis revealed an interaction between CH and TPP in the nanoparticle system.

The positive results obtained by physical–chemical characterization of these systems confirmed their high potential as drug carriers. Additionally, the possibility of applying ultrasound only to the CH dispersion demonstrated how to preserve drug loading and maintain the best features of both dimension and polydispersity of CH nanoparticles. These features might be beneficial for the loading of sensitive molecules such as proteins.

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