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Ultrasonication of chitosan and chitosan nanoparticles

E.S.K. Tang, M. Huang, L.Y. Lim*

Department of Pharmacy, National University of Singapore, 18, Science Drive 4, Singapore 117543, Singapore

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Abstract

The objective of this study was to evaluate the effects of ultrasonication on chitosan molecules and nanoparticles. Molecular weight (M_v) of chitosan HCl (M_v 146 kDa and degree of deacetylation (DD) 96%) decreased linearly with increasing duration and amplitude of ultrasonication. DD and FTIR absorption were unaffected. X-ray diffraction (XRD) analysis suggested greater chain alignment in the ultrasonicated chitosan samples. Chitosan nanoparticles had mean diameter of 382 nm, polydispersity of 0.53 and zeta potential of 47 mV. Ultrasonication administered at increasing duration or amplitude decreased the mean diameter and polydispersity of the nanoparticles. Zeta potential and FTIR absorbance were unaffected, while XRD suggested a greater disarray of chain alignment in the nanoparticle matrix. Under the transmission electron microscope (TEM), freshly prepared nanoparticles were dense spherical structures which became fragmented after ultrasonication for 10 min at amplitude of 80. Untreated nanoparticle formulation turned turbid upon storage for 3 weeks at ambient conditions due to substantial swelling of the nanoparticles. Ultrasonicated nanoparticle formulation remained clear on storage. Although the particles had also swelled, they were no longer spherical, assuming instead an irregular shape with branching arms. In conclusion, high-intensity ultrasonication induced considerable damage on the chitosan nanoparticles which could affect their function as drug carriers.

Keywords: Chitosan nanoparticles; Ultrasonic; M_v; Particle size; TEM; Storage

1. Introduction

Chitosan [α (1 → 4) 2-amino 2-deoxy β-D-glucan] is a linear polyamine with a high ratio of glucosamine to acetyl-glucosamine units. The percentage of glucosamine units in the polymer is known as its degree of deacetylation (DD) (Paul and Sharma, 2000). Protonation of the amino group allows the polymer to be solubilized in aqueous acids and to interact with negatively charged materials (Suheyla, 1997). It is this functional group that enables the formation of chitosan nanoparticles by crosslinking and desolvation with cationic salts (Janes et al., 2001). Chitosan nanoparticles are attractive non-viral carriers for the delivery of peptides, proteins, oligonucleotides, and plasmids (Janes et al., 2001). They have the capacity to protect sensitive bioactive macromolecules from enzymatic and chemical degradation in vivo and during storage (Mao et al., 2001), and to facilitate the transport of charged macromolecules across absorptive epithelial cells (Takeuchi 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 in reducing the size and polydispersity of nanoparticles (Grieser et al., 1999). The physical stability and

* Corresponding author. Tel.: +65-6874-6137; fax: +65-6779-1554.
E-mail address: phalimly@nus.edu.sg (L.Y. Lim).

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in vivo distribution of nanoparticles are affected by their mean size, polydispersity, and surface charge density (Bodmeier and Maincent, 1996). Despite the wide-spread applications of ultrasonication in nanotechnology, its effects on chitosan nanoparticles are not well understood, although there have been several reports on the ultrasonication of the chitosan polymer. It is generally agreed that ultrasonication causes main chain scissions at the 1,4-glycosidic bond (Chen et al., 1997) without affecting the DD of chitosan samples. The process has, therefore, been conveniently applied to produce chitosan samples of lower molecular weights of the same DD (Signini et al., 2000; Tsaih and Chen, 1999; Chen and Hwa, 1996). We hypothesized that ultrasonic-mediated depolymerization of chitosan would influence the properties of the chitosan nanoparticles. The complexity of the chitosan nanoparticle system may further render it more vulnerable to chemical modifications by ultrasonication.

High-intensity ultrasonication produces acoustic cavitation, which generates hot spots of short lifetimes with intense local heating of ~5000 °C, pressures of ~1000 atm, and heating and cooling rates above 10^{10} K/s (Suslick et al., 1999). These, together with free-radical formation, may mediate redox reactions and intramolecular regroupings in the samples (El'Piner, 1964). Cavitation also generates rapid streaming of solvent molecules around the cavitation bubble, as well as shock waves during bubble collapse, which in turn generate very large shear forces (Suslick and Price, 1999). In addition, rarefractions and compressions of the liquid media can cause dispersive (particle separation) and coagulative (collision and adhesion of particles) phenomena, respectively (Carlin, 1960).

The objective of this project was to evaluate and correlate the effects of ultrasonication on the properties of chitosan in solution and chitosan nanoparticles. Changes in the physicochemical properties of chitosan were monitored by analyses of its molecular weight (M_v), DD, Fourier transform infra-red (FTIR) spectrum, and X-ray diffraction (XRD). Ultrasonic-mediated changes in the mean diameter, polydispersity, zeta potential, FTIR spectrum, and XRD of the chitosan nanoparticles were determined. The morphology of the chitosan nanoparticles was also observed.

2. Materials and methods

2.1. Materials

Materials used were medical grade chitosan hydrochloride (Protasan™, Pronova Biomedical, Norway), pentasodium tripolyphosphate or TPP (Merck, Germany), CH_3COOH (BDH Laboratories, UK), CH_3COONa (Merck, Germany), and tungstophosphoric acid (Merck, Germany).

2.2. Processing by ultrasonication

Chitosan nanoparticles were prepared by ionotropic gelation by adding 8 ml of 0.10% TPP in water to 16 ml of 0.25% of Protasan™ in water at a stirring speed of 1000 rpm (MR3001, Heidolph, Germany). Chitosan solutions were prepared by adding 8 ml of water to 16 ml of 0.25% of Protasan™ in water. All samples were ultrasonicated immediately after preparation.

Ultrasonic treatments were administered using an ultrasonic probe with diameter of 3 mm and a 130 W high-intensity ultrasonic processor (VC130, Sonics and Materials Inc., USA) operating at 20 kHz. The converter was made of piezoelectric lead zirconate crystals. Samples (24 ml) in glass universal bottles (Beatson and Co., UK) were equilibrated to 25 °C and ultrasonicated under continuous mode at ambient conditions. The probe was immersed 4 cm into the sample during ultrasonication, which was carried out at specified amplitudes (20, 40, 60, 80) over durations of 2–10 min. For simplicity, the ultrasonication conditions are denoted as *AxTy* where *x* represents the amplitude and *y* the duration. Amplitudes of 20, 40, 60, and 80 corresponded to intensities of approximately 14, 42, 70, and 99 W/cm^2, respectively, the intensity calculated by taking the difference between the output Watts delivered into the sample and in air, divided by the area of the probe tip.

Ultrasonication produced similar heating effects in the chitosan solution and chitosan nanoparticle samples. In both cases, temperature increased linearly from 25 to 45 °C for samples ultrasonicated for 5 min at increasing amplitudes from 0 to 80, and from 25 to 41 °C for samples ultrasonicated at the amplitude of 40 with increasing duration from 0 to 10 min. Treated samples were cooled to ambient temperature and analyzed immediately. Some samples were

lyophilized (FD3, Dynavac Engineering, Australia) before analysis.

2.3. Characterization of chitosan molecules and nanoparticles

Molecular weight of chitosan was determined in triplicates by dilute solution capillary viscometry at 30 ± 0.05 °C using a Cannon Ubbelohde four bulb shear dilution viscometer. Filtered chitosan solutions (0.05–0.2% in 0.2 M CH_3COOH/0.1 M CH_3COONa) were equilibrated to 30 °C prior to measurement of flow times. The intrinsic viscosity was determined by linear regression of the graph of reduced viscosity against concentration ($R^2 > 0.96$). The viscosity average M_v was calculated from the intrinsic viscosity $[\eta]$ using the Mark–Houwink equation ($[\eta] = kM_v^{\alpha}$). The k and α values were 6.589×10^{-3} and 0.88, respectively (Wang et al., 1991).

DD of chitosan was determined by the first derivative UV-spectrophotometric method (Tan et al., 1998) using 0.10 mg/ml of chitosan in 0.01 M CH_3COOH and a UV absorbance range of 190–250 nm. FTIR spectra were obtained (FT/IR-430 Spectrometer, JASCO, Japan) with 200-mg KBr disks containing 0.1% of freeze-dried sample. X-ray diffractograms (D5005 X-ray diffractometer, Siemens, Germany) of a thin layer of freeze-dried sample were acquired in the range of $5^\circ < 2\theta < 30^\circ$ at a scan rate of 1.2°/min.

Particle diameter, zeta potential, and polydispersity of the chitosan nanoparticles were measured using a particle sizer (Zetasizer 3000HS_A, Malvern Instruments, UK). The polydispersity is a measure of the size distribution of the nanoparticles. The morphology of the chitosan nanoparticles was observed under a TEM (100CXII, JEOL, Japan) after staining with 2% of tungstophosphoric acid. Samples were observed immediately after ultrasonication and after 3 weeks of storage at ambient conditions post-ultrasonication.

2.4. Statistical analyses

Data are presented as mean ± S.D. Data on M_v, DD, mean diameter, zeta potential, and polydispersity were analyzed by one-way ANOVA with post hoc tests of least significant difference (SPSS 10.0, $P = 0.05$). A 2×2 factorial design ($P = 0.05$) was employed to determine the relative contributions of the amplitude (40 and 80) and duration (5 and 10 min) of ultrasonication on M_v and mean particle diameter.

3. Results

Protasan™ had a viscosity average M_v of 145.95 ± 6.74 kDa and a DD of 96.25 ± 0.25% (Table 1). M_v of the sample decreased to 136.70 kDa after ultrasonication at A20T5 and further to 122.03 kDa when treated at A80T5 (Fig. 1a). Samples treated for 5 min showed a linear relationship ($R^2 = 0.95$) between M_v and amplitude. The duration of ultrasonication was also important, for the M_v decreased linearly from 136.79 to 113.38 kDa ($R^2 = 0.99$) when treatment was prolonged from A40T2 to A40T10 (Fig. 1b). Factorial analysis showed M_v to be decreased by 7.49 kDa when the ultrasonication amplitude was increased from 40 to 80, and by 16.13 kDa when ultrasonication was prolonged from 5 to 10 min. The interaction effect between the two parameters was 0.08 kDa. Analysis by one-way ANOVA using $F_{0.05}(1,8)$ suggested that the ultrasonication duration, not the amplitude, had a significant impact on M_v. There was a lack of significant interaction between the two factors.

DD of the chitosan samples did not vary significantly with the range of durations and amplitudes of ultrasonication employed in this study (Table 1). Comparable FTIR spectra (Fig. 2) were obtained after the chitosan samples were ultrasonicated at the harshest condition of A80T10, suggesting the absence of chemical modifications. The major IR absorption bands were attributed to O–H stretch (3430 cm^{-1}), $N–H_3^+$ stretch (2100 cm^{-1}), N–H bend (1630 cm^{-1}),

Table 1
Molecular weight (M_v) and degree of deacetylation (DD) of chitosan samples as a function of the amplitude and duration of ultrasonication

Amplitude	Duration (min)	DD (%)	M_v (kDa)
0	0	96.25 ± 0.25	145.95 ± 6.74
20	5	96.37 ± 0.26	136.70 ± 4.70
40	5	96.38 ± 0.04	129.60 ± 8.18
40	2	96.06 ± 0.05	136.79 ± 4.85
40	10	96.34 ± 0.14	113.38 ± 9.65
80	5	96.08 ± 0.10	122.03 ± 5.85
80	10	96.47 ± 0.07	105.98 ± 4.27

Mean ± S.D., $n = 3$.

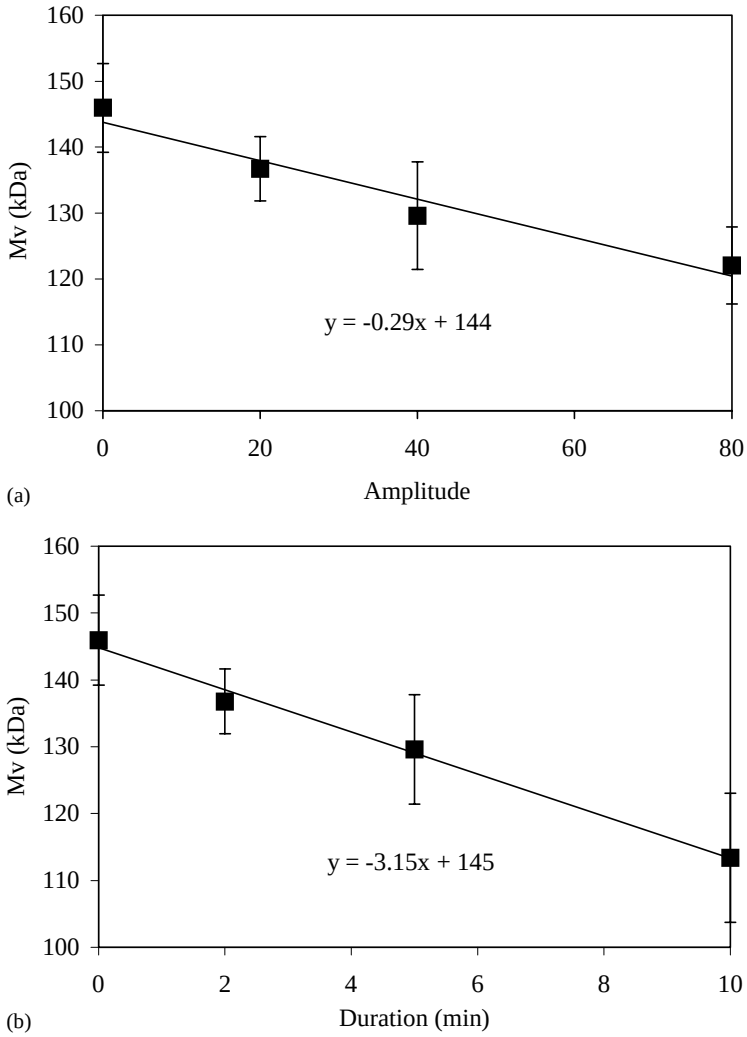

Fig. 1. Molecular weight of chitosan as a function of (a) amplitude and (b) duration of ultrasonication. Samples in panel (a) were processed for 5 min, while those in panel (b) were processed at an amplitude of 40 (mean ± S.D., $n = 3$).

and C–O stretch (1100 cm^{-1}). Re-alignment of polymer chains were, however, implicated by the XRD (Fig. 3) of chitosan samples ultrasonicated at A80T10, which showed a considerable sharpening of the main peak in the 2θ range of 20–25°.

The chitosan nanoparticles had mean diameter of 382 ± 4 nm, polydispersity of 0.53 ± 0.05, and zeta potential of 47.48 ± 1.32 mV (Table 2). They produced an FITR spectrum (Fig. 4) similar to that of the parent polymer (Fig. 2). Nanoparticles ultrasonicated for 5 min showed decreasing mean diameter with increasing amplitude of ultrasonication (Fig. 5a), but the rate of decrease in particle size was not uniform over the range of amplitudes investigated. A leveling effect was apparent at amplitudes higher than 60. Likewise, nanoparticles ultrasonicated at amplitude of 40 exhibited decreasing particle size with increasing duration of treatment, the particle size reaching a limiting value at treatment duration ≥8 min (Fig. 5b). There was no significant difference in the mean sizes of nanoparticles ultrasonicated for 8 and 10 min. Parallel trends were observed for the polydispersity of the chitosan

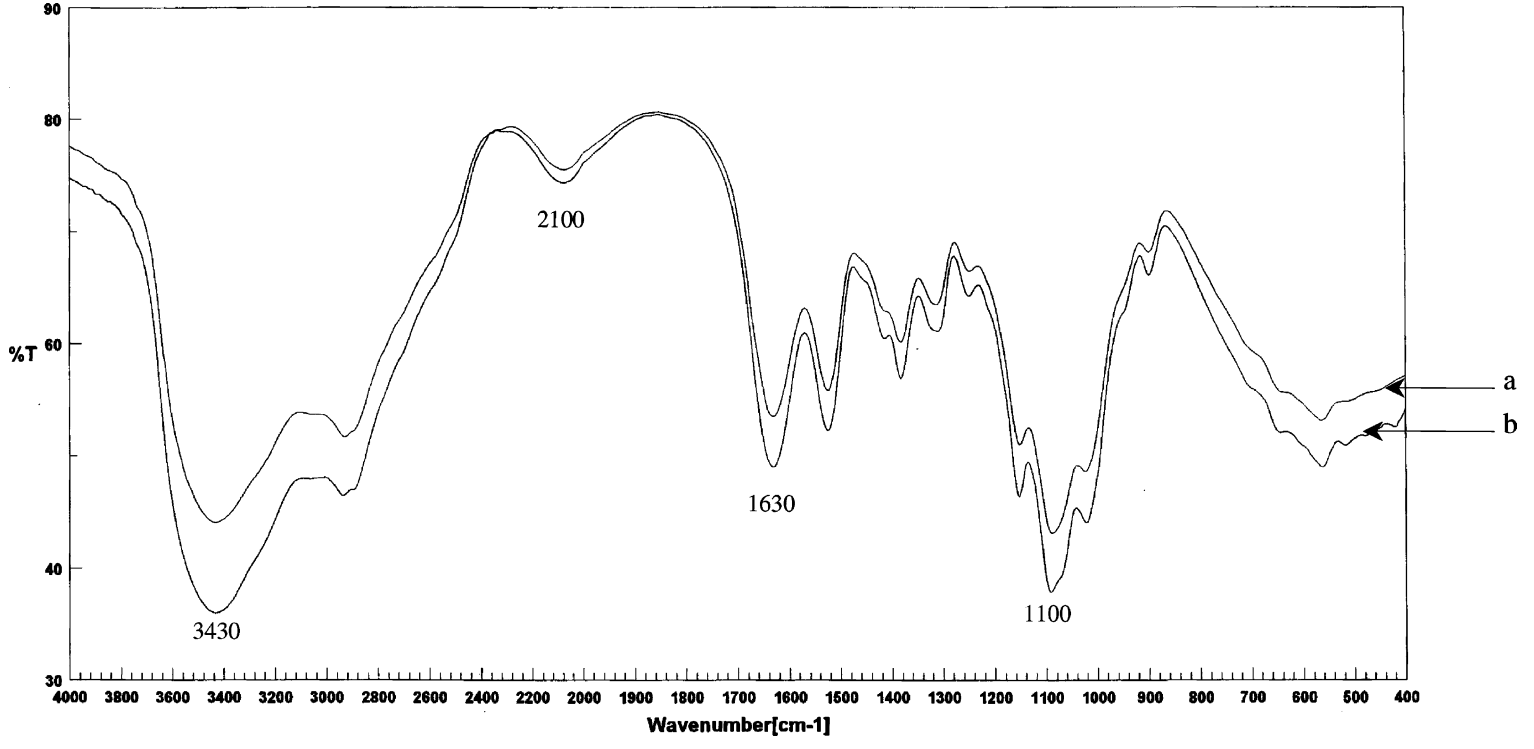

Fig. 2. FTIR spectrum of chitosan (a) before and (b) after ultrasonication for 10 min at an amplitude of 80.

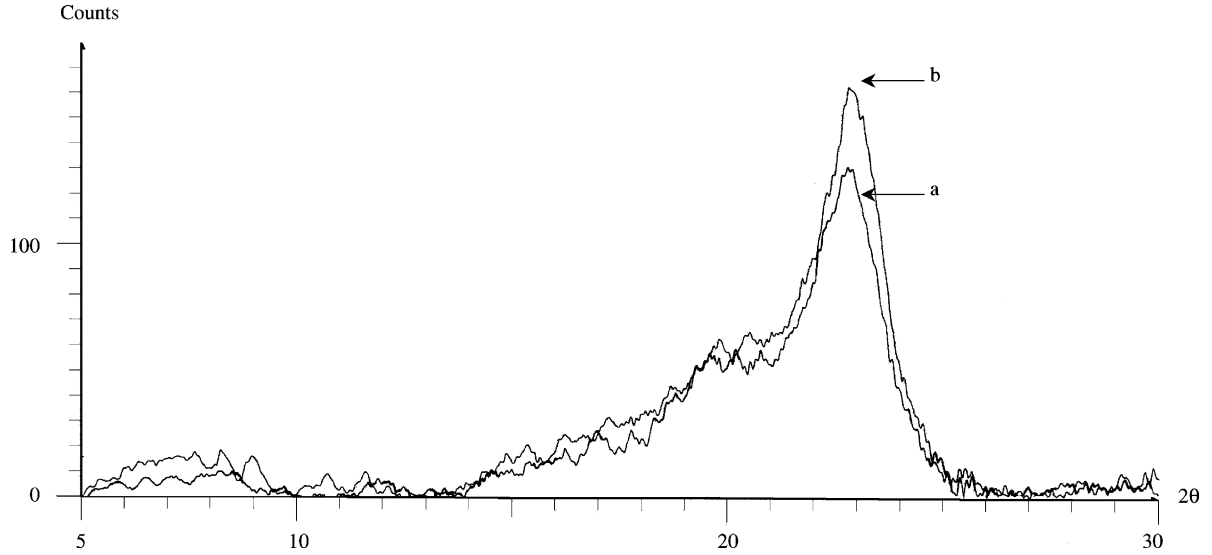

Fig. 3. X-ray diffractogram of chitosan (a) before and (b) after ultrasonication at an amplitude of 80 for 10 min.

nanoparticles as a function of the amplitude and duration of ultrasonication (Fig. 6). In both cases, a leveling off of the polydispersity value was observed at high amplitude and duration of ultrasonication.

Factorial analysis indicated a decrease in the mean particle size by 23 nm when the ultrasonication amplitude was increased from 40 to 80, while particle size decreased by 19 nm when the ultrasonication duration was prolonged from 5 to 10 min. Interaction effect was 0.05 nm. Analysis by one-way ANOVA using $F_{0.05}(1,8)$ found both the ultrasonication amplitude and duration to have significant influences on the size of the chitosan nanoparticles. However, there was no significant interaction between the two factors.

Table 2
Characteristics of chitosan nanoparticles following ultrasonication at specified amplitude and duration

Amplitude	Duration (min)	Mean diameter (nm)	Polydispersity	Zeta potential (mV)
0	0	382 ± 4	0.53 ± 0.05	47.48 ± 1.32
20	5	350 ± 3	0.37 ± 0.05	46.51 ± 0.22
40	5	325 ± 1	0.36 ± 0.03	46.02 ± 1.02
60	5	314 ± 1	0.34 ± 0.02	46.09 ± 0.15
80	5	302 ± 2	0.31 ± 0.02	46.05 ± 0.21
40	2	346 ± 1	0.40 ± 0.04	46.06 ± 0.24
40	8	312 ± 2	0.32 ± 0.04	46.36 ± 0.44
40	10	306 ± 3	0.31 ± 0.02	45.34 ± 0.22
80	10	283 ± 3	0.30 ± 0.01	45.51 ± 0.29

Mean ± S.D., $n = 3$.

The zeta potential of the nanoparticles did not change significantly following ultrasonication at even the harshest condition of A80T10 (Table 2), indicating that the surface charge density of the nanoparticles was preserved. Like the parent polymer, the FTIR spectrum of the ultrasonicated chitosan nanoparticles was comparable to that of the untreated sample (Fig. 4), again implicating the absence of apparent chemical changes. On the other hand, ultrasonication produced an XRD effect in the chitosan nanoparticles opposite to that seen in the parent polymer (Fig. 7). Unlike the chitosan polymer, the ultrasonicated (at A80T10) chitosan nanoparticles exhibited a peak of lower amplitude in the 2θ range of 20–25°, suggesting increased disarray in chain alignment compared to the untreated nanoparticles.

The freshly prepared chitosan nanoparticle formulation was a translucent liquid. Under the TEM, the sample appeared as discrete particles and aggregates of various sizes (Fig. 8a and b), all of which presented with a dense structure. Ultrasonication at A80T10 did not affect the gross appearance of the formulation, but TEM micrographs showed disruption of nanoparticle structure and the production of smaller fragmented particles (Fig. 8c and d). After 3 weeks of storage at ambient conditions, the untreated

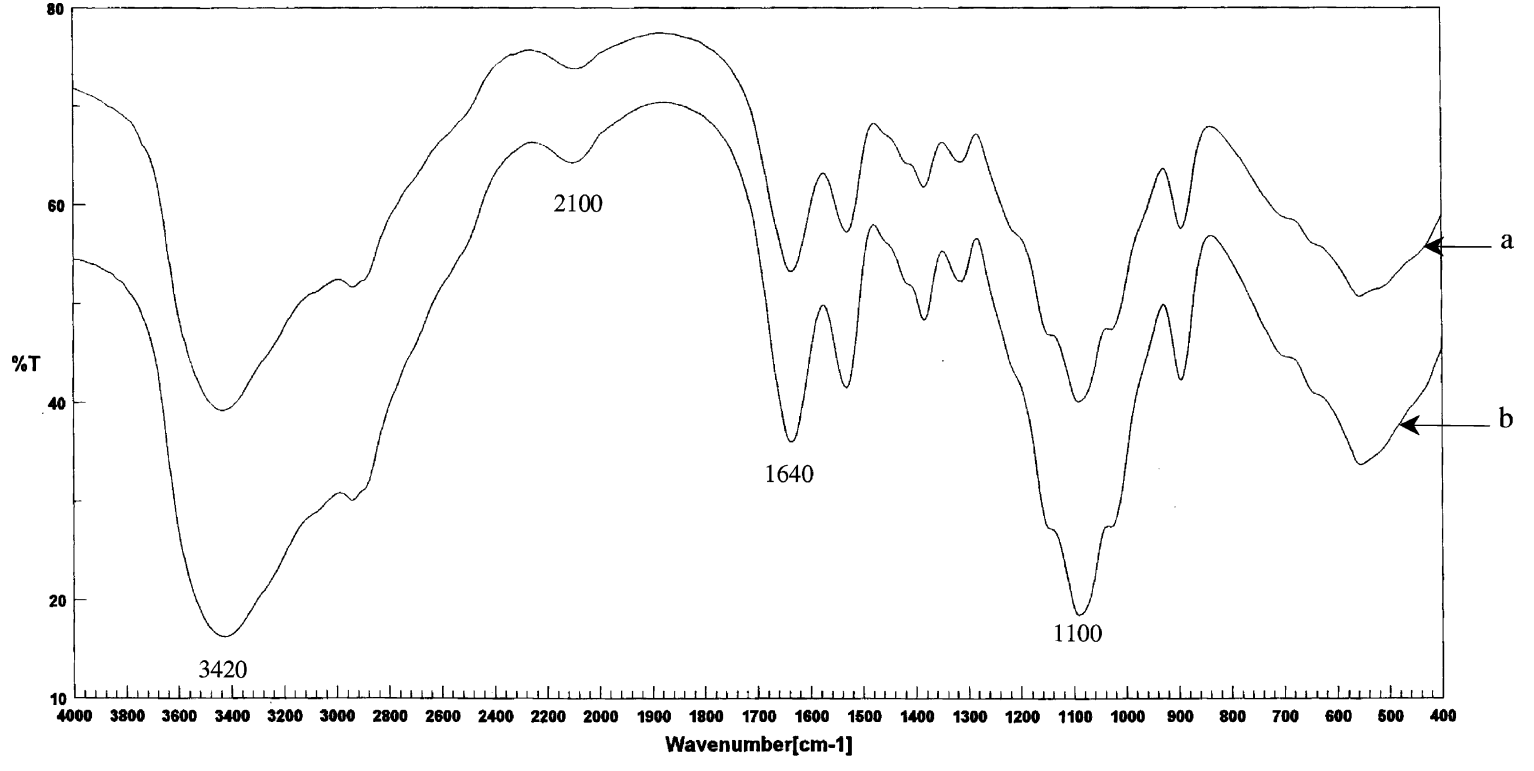

Fig. 4. FTIR spectrum of chitosan nanoparticles (a) before and (b) after ultrasonication for 10 min at an amplitude of 80.

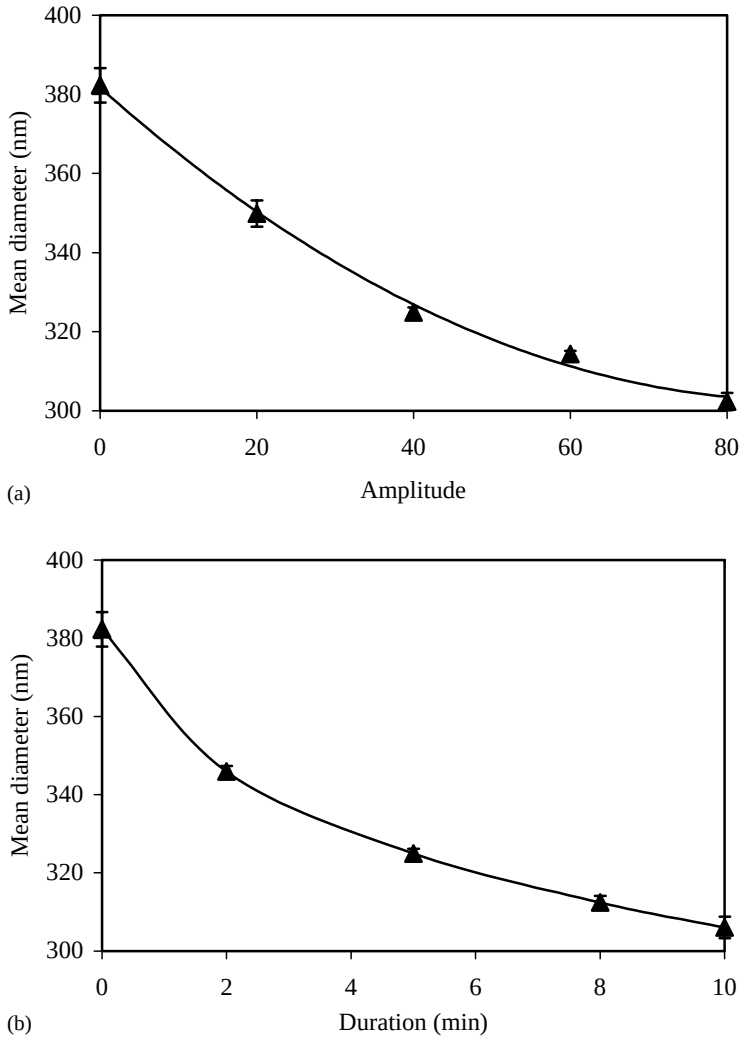

Fig. 5. Mean particle diameter of chitosan nanoparticles as a function of (a) amplitude and (b) duration of ultrasonication. Samples in panel (a) were processed for 5 min, while those in panel (b) were processed at an amplitude of 40 (mean ± S.D., $n = 3$).

formulation turned turbid, the nanoparticles having become enlarged spheres with a fractured dense outer layer (Fig. 8e). Although turbidity was not observed of formulations similarly stored after ultrasonication at A80T10, the nanoparticles had also undergone morphological changes. The particles no longer retained a spherical structure but appeared as irregular particles with radiating branches (Fig. 8f).

4. Discussion

Ultrasonication of Protasan^{TM} in solution under the harshest condition of A80T10 produced a 27% reduction in M_v, the duration of ultrasonication having a more dominant influence than the amplitude of ultrasonication on the M_v. These data correlated with literature reports in demonstrating the depolymerization of chitosan after ultrasonication (Chen et al., 1997;

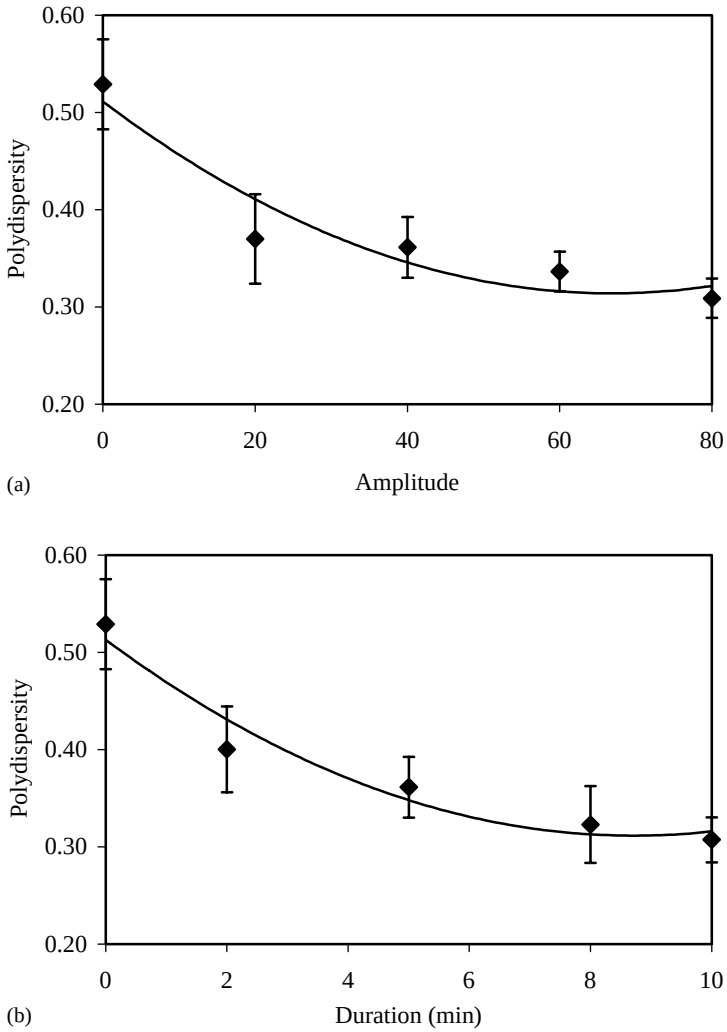

Fig. 6. Polydispersity of chitosan nanoparticles as a function of (a) amplitude and (b) duration of ultrasonication. Samples in panel (a) were processed for 5 min, while those in panel (b) were processed at an amplitude of 40 (mean ± S.D., $n = 3$).

Signini et al., 2000; Tsaih and Chen, 1999; Chen and Hwa, 1996). Depolymerization is postulated to occur by main chain scission of the 1,4-glycosidic bond, probably through the high shear forces generated during ultrasonication. Although heat was also generated during ultrasonication, we did not notice any exceptionally large increase in the temperature of the bulk samples. Moreover, there is conflicting evidence as to whether intense local heating could contribute to polymer depolymerization by main chain scissions (Price, 1999; Chen and Tsaih, 1998).

The reduced particle size of the ultrasonicated chitosan nanoparticles was probably related to the depolymerization of chitosan molecules. This is because the percent change in the mean particle diameter of the nanoparticles as a function of the amplitude and duration of ultrasonication were comparable to the percent change in M_v of the parent polymer. In addition, TEM micrographs showed fragmentation of the nanoparticles after ultrasonication. Both the amplitude and duration of ultrasonication were important in decreasing the size and polydispersity of the chitosan nanoparti-

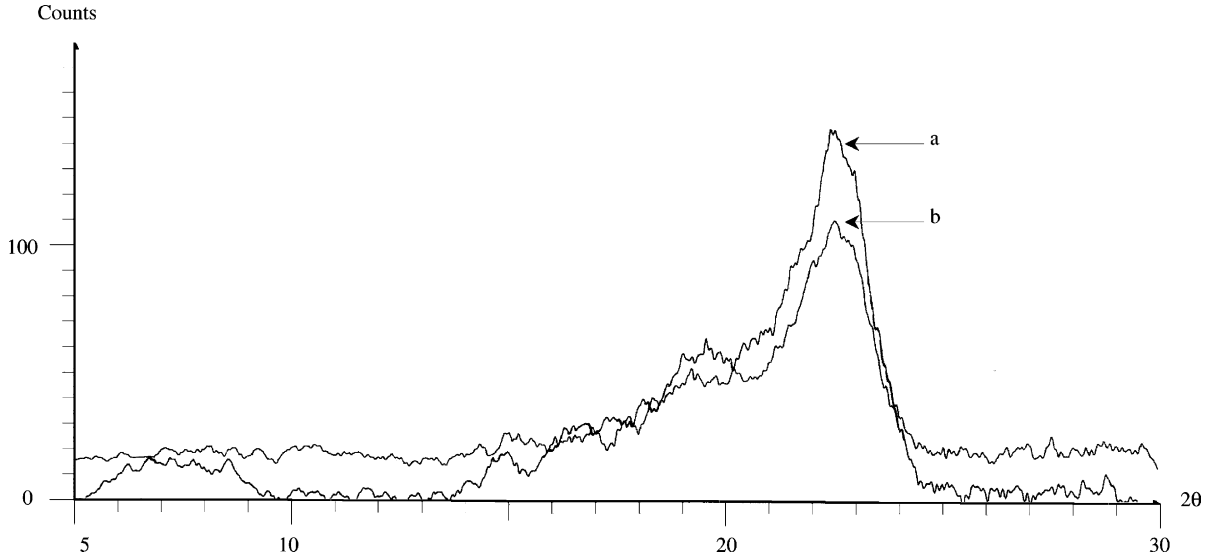

Fig. 7. X-ray diffractograms of chitosan nanoparticles (a) before and (b) after ultrasonication for 10 min at an amplitude of 80.

cles. However, the rate of size reduction slowed down considerably to reach a limiting diameter of ∼300 nm when ultrasonication was carried out beyond the duration of 8 min or beyond the amplitude of 60. Increasing amplitude might create more cavitation, which dampened the efficiency of energy transmission and reduced the ultrasonic effect (Mason, 1999). The leveling effect could also be associated with the decreased absorption coefficient and increased relaxation of the shorter chitosan polymer chains (Lii et al., 1999). The polydispersity profiles have suggested that the larger nanoparticles were more susceptible to the ultrasonication effects than the smaller nanoparticles.

Other than depolymerization, ultrasonication did not appear to cause any other chemical modifications based on the IR absorption of the chitosan samples. DD of the polymer was also unchanged. While it might be argued that it would be difficult to raise the DD of a chitosan sample with a high DD of 96%, we note that ultrasonication also had no effect on the DD of a chitosan sample with a lower DD of 77% (Signini et al., 2000). Based on its effect on the DD, it is not surprising that ultrasonication did not modify the surface charge properties of the chitosan nanoparticles.

The XRD of chitosan is characteristic of an amorphous polymer. Ultrasonication followed by freeze-drying appeared to lead to greater chain alignment in the polymer network structure. Underlying this structural modification were possibly ultrasonic-mediated changes in the way the chitosan chains developed intermolecular and intramolecular hydrogen bonds, with chain realignment facilitated after main chain scissions. Chain realignment was, however, not observed in similarly processed chitosan nanoparticles. Chitosan nanoparticles comprised of a dense network structure of interpenetrating polymer chains crosslinked to each other by TPP counterions. Although ultrasonication might truncate the chitosan molecules to yield shorter, flexible chains, the crosslinks hindered significant chain mobility and realignment. Conversely, the XRD implicated greater disarray in chain alignment in the nanoparticles after ultrasonication and freeze-drying. Of greater concern is that the chain scissions created by ultrasonication might weaken the integrity of the polymer matrix to give rise to friable particles that fragment readily. Particle fragmentation, evident from the TEM micrographs of ultrasonicated samples, is undesirable because it adversely affects the drug loading capacity and in vivo performance of the nanoparticles.

Another important finding was the effects of storage on the characteristics of the chitosan nanoparticles. Storage for 3 weeks at ambient conditions turned the translucent nanoparticle formulation into a turbid liq-

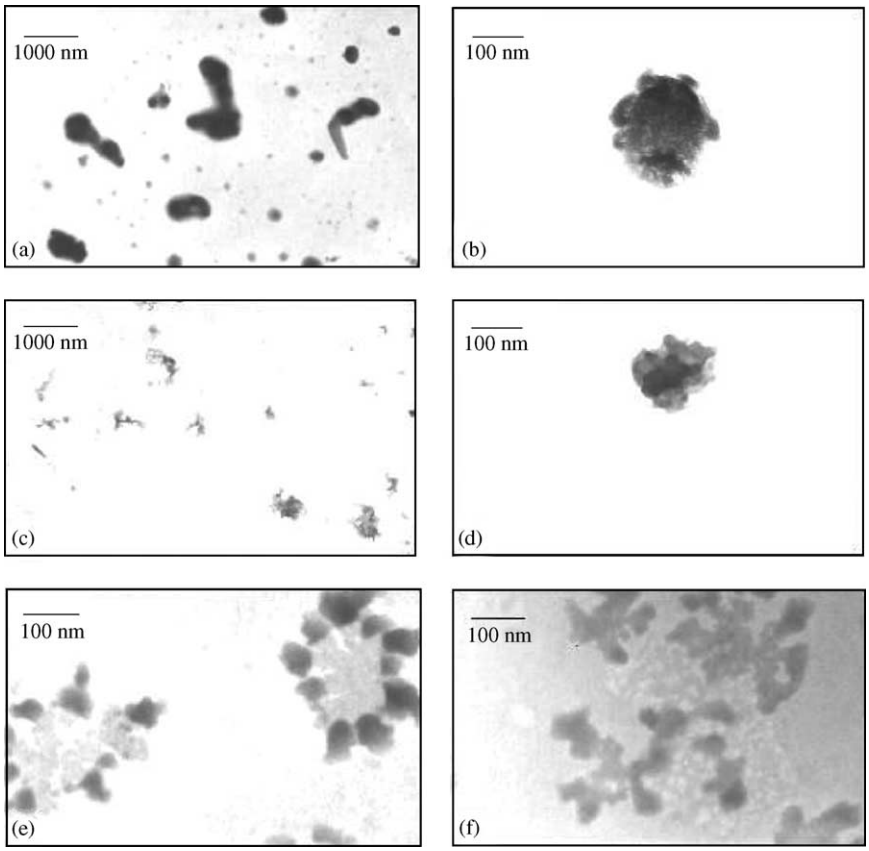

Fig. 8. TEM micrographs of freshly prepared chitosan nanoparticles at magnification of $10^4\times$ (a) and $10^5\times$ (b); freshly prepared nanoparticles ultrasonicated at an amplitude of 80 for 10 min at magnification of $10^4\times$ (c) and $10^5\times$ (d); untreated nanoparticles stored for 3 weeks at ambient conditions ($10^5\times$) (e), and ultrasonicated nanoparticles stored for 3 weeks at ambient conditions ($10^5\times$) (f).

uid. TEM micrographs associated the turbidity with a growth in particle mean size, contributed possibly by an inflow of water into the nanoparticles by osmosis due to the presence of TPP. This caused the nanoparticle to expand and the polymer matrix to fracture. Nevertheless, the spherical shape was maintained despite the swelling of some of the particles to a size readily discernible by the naked eye. In contrast, nanoparticle samples ultrasonicated at A80T10 remained translucent after 3 weeks of storage at ambient conditions. Viewed under the TEM, the individual nanoparticles had also become larger but there was a concomitant loss of sphericity which might be attributed to the fragmentation of the nanoparticles prior to storage. Upon swelling on storage, the fragmented matrix radiated out as flagging branches, their flexibility ensuring that the resultant hydrodynamic radius was not significantly increased as to give the formulation a turbid appearance.

5. Conclusion

Ultrasonication caused the depolymerization of chitosan in solution but did not appear to induce any other chemical modifications. DD of the polymer was unchanged. The shorter chains produced by ultrasonication showed a higher degree of chain alignment when the polymer was subsequent freeze-dried. The duration of ultrasonication played a more dominant role in controlling the chain length of the chitosan polymer compared to the amplitude of ultrasonication. Ultrasonication also decreased the mean particle diameter and polydispersity of the chitosan nanoparti-

cles, but did not affect the zeta potential. The changes in particle size and polydispersity could have resulted from ultrasonic-mediated depolymerization of chitosan chains. The ultrasonicated nanoparticles were friable and fragmented, and there was greater disarray in chain realignment when the nanoparticles were freeze-dried. Storage exacerbated the damage, the ultrasonicated nanoparticles becoming irregularly shaped structures of radiating branches after 3 weeks of storage at ambient conditions. It is therefore not advisable to use high-intensity ultrasonication to prepare or process chitosan nanoparticles for drug delivery.

Acknowledgements

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