Preparation and properties of ionically cross-linked chitosan nanoparticles

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Chitosan nanoparticles were fabricated by a method of tripolyphosphate (TPP) cross-linking. The influence of fabrication conditions on the physical properties and drug loading and release properties was investigated by transmission electron microscopy (TEM), dynamic light scattering (DLS), and UV–vis spectroscopy. The nanoparticles could be prepared only within a zone of appropriate chitosan and TPP concentrations. The particle size and surface zeta potential can be manipulated by variation of the fabrication conditions such as chitosan/TPP ratio and concentration, solution pH and salt addition. TEM observation revealed a core–shell structure for the as-prepared nanoparticles, but a filled structure for the ciprofloxacin (CH) loaded particles. Results show that the chitosan nanoparticles were rather stable and no cytotoxicity of the chitosan nanoparticles was found in an in vitro cell culture experiment. Loading and release of CH can be modulated by the environmental factors such as solution pH and medium quality. Copyright © 2008 John Wiley & Sons, Ltd.

\textbf{Keywords:} chitosan; nanoparticles; ionic crosslinking; properties; drug delivery

\section*{INTRODUCTION}

Drug delivery systems are an effective means to realize the sustained release of many kinds of drugs. Chitosan and its derivative materials, in particular with a range of particles, have been diversely employed in the field of drug delivery. More recently, chitosan nanoparticles have attracted much attention by virtue of their large drug loading capacity, good adsorption performance, and long shelf life. Several techniques have been developed to prepare chitosan nanoparticles, such as emulsion cross-linking,\cite{1, 2} ionic gelation,\cite{3–5} spray drying,\cite{6} and so on.

Chitosan contains abundant amino and hydroxyl groups, which enable nanoparticle formulation via both physical and chemical cross-linking.\cite{7} Covalent cross-linking is usually achieved by treatment of glutaraldehyde, which reacts with the amino groups to form Schiff bases. Ionic cross-linking of chitosan is a typical non-covalent interaction, which can be realized by association with negatively charged multivalent ions such as tripolyphosphate (TPP).\cite{8, 9} For pharmaceutical applications, physical cross-linking is more promising since the cross-linking is reversible and may largely avoid potential toxicity of the reagents.

Although diverse efforts have been made to obtain the chitosan nanoparticles via TPP cross-linking following the pioneering work of Calvo et al.\cite{10} and to explore the potential pharmaceutical applications,\cite{11–14} optimization of the fabricating conditions and the comprehensive properties of the resultant chitosan nanoparticles is still an ongoing important topic in this field. These parameters not only affect the storage stability, loading, and release performance but also govern the interaction of the particles with different biological tissues where they are introduced. In this context, special attention is paid to the drug delivery and release properties.\cite{10, 15, 16} The effects of pH of TPP solution, TPP concentration, and ionic strength on the entrapment efficiency, release, and activity of lipase in chitosan hydrogel beads were also studied.\cite{17} However, there are many discrepancies in terms of the alteration principles versus fabrication conditions, and explanation of the observed phenomena.\cite{18, 19} Moreover, little attention is paid to the storage stability and biocompatibility of the chitosan nanoparticles, which are critical issues for practical applications in the pharmaceutical field.

In this work, the influence of fabrication conditions is systematically investigated. Stability and biocompatibility of the prepared chitosan nanoparticles are assessed. Loading and release of a model drug, ciprofloxacin (CH), is also performed to testify the potential application of these nanoparticles. CH is a kind of antiseptic, which has an effective anti-bacterial effect against both Gram-positive and negative bacteria. To reduce the
drug toxicity as a result of long term application, for example arthritis damage for children, various sustained release carriers such as microcapsules, gels and liposomes have been developed. Yet so far less attention has been given to the nanoparticle carriers, in particular the chitosan nanoparticles.

**EXPERIMENTAL**

**Materials**

Chitosan (CS, $M_w = 620$ kDa, degree of deacetylation $= 90\%$, viscosity $= 115$ cps) is a commercial product of Haidebei Halobios Co., Ltd. (Jinan, China). Sodium tripolyphosphate (TPP) was purchased from Dongsheng Chemical Reagent Factory (Wenzhou, China). Ciprofloxacin hydrochloride (CH, $C_{18}H_{21}FN_2O_3\cdot HCl\cdot H_2O$) was purchased from Zhejiang Medicine Co. Ltd., Xinchang Pharmaceutical Factory (Shaoxing, China). Phosphotungstic acid was purchased from Sinopharm Group Chemical Reagent Co., Ltd. (Shanghai, China). All other chemicals were of analytical grade and used as received. The water used in all experiments was triple distilled.

**Preparation of the chitosan nanoparticles**

The nanoparticles were obtained based on ionic gelation of TPP with chitosan. Briefly, chitosan and TPP were dissolved in acetic acid and triple distilled water to obtain solutions of 1.0–5.0 and 0.25–2.0 mg/ml, respectively. The chitosan nanoparticles were obtained upon addition of 14 ml TPP solution into 35 ml chitosan solution under mild mechanical stirring (550 rpm) at room temperature. The drug-loaded nanoparticles were similarly prepared by using a chitosan solution containing the drug.

**Characterization**

Morphological examination of the chitosan nanoparticles was performed by transmission electron microscopy (TEM) (JEOL JEM-200CX, Japan). The sample was stained with 2% (w/v) phosphotungstic acid and a drop of the sample was applied onto a carbon-coated copper meshwork, and dried at room temperature. The acceleration voltage was 100 kV. Zeta potential of the particles was recorded on a Zetasizer 2000. Each value was averaged from three parallel measurements.

The particle size was measured by dynamic light scattering (DLS) (90 Plus/BI-MAS). The wavelength of laser is 658 nm. The nanoparticle suspensions were stored at 4°C and the sizes of the nanoparticles were monitored by DLS.

**Stability of the chitosan nanoparticles**

The stability of the chitosan nanoparticles obtained at different fabricating conditions was examined in terms of the particle size. The nanoparticle suspensions were stored at 4°C and the sizes of the nanoparticles were monitored by DLS.

**Biocompatibility of the chitosan nanoparticles**

In vitro cell culture was performed to determine the biocompatibility of the as-prepared chitosan nanoparticles. The chitosan nanoparticles were sterilized by UV irrigation before cell culture. Human dermal fibroblasts were isolated from foreskins and routinely expanded. The cells were cultured at 37°C, in 5% CO2, and 95% humidity in DMEM supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml), and 10% fetal bovine serum (FBS) (complete medium), which was changed every 3 days. Cells were passaged at confluence and the 4–8th passage of fibroblasts with a density of $2 \times 10^5$/ml were seeded in a 96-well polystyrene plate. After 24 hr, 60 μl of nanoparticle suspension was added into each well. The final nanoparticle concentration was high enough for close contact between the cells and the nanoparticles. The culture medium was changed every 2 days. Meanwhile, normal culture medium was used in parallel as a negative control. The cell proliferation was measured by methylthiazoletetrazolium (MTT) assay. The absorbance was recorded at a wavelength of 570 nm by a microplate reader (Bio-Rad model 550). Three parallel samples were analysed and data were expressed as mean ± standard deviation.

**Quantification of the relative CH amount in the chitosan nanoparticles**

The prepared nanoparticle suspension containing the CH was centrifugated at 12,000 rpm for 70 min to obtain the supernatant, which was diluted over hundreds of times and the absorbance at 276 nm was recorded by a UV–vis spectrophotometer (Shimadzu UV-2550). The supernatant of blank nanoparticles was obtained under the same conditions and used as a control. The concentration of CH in the supernatant was quantified by referring to a calibration curve recorded from a known amount of CH at the same condition. A lower concentration found in the supernatant implies a higher loading efficiency in the chitosan nanoparticles.

**Release of CH**

CH release was performed using a dialysis technique. The CH loaded chitosan nanoparticles were centrifugated at 12,000 rpm for 70 min to obtain the nanoparticle precipitate, which was washed with water twice. The nanoparticles were transferred into
a dialytic-bag with a cut-off molecular weight of 3.5 kDa. The dialytic-bag was then immersed into 100 ml phosphate buffered saline (PBS, pH 7.4) or H₂O at 37°C under continuous shaking. At predetermined time, 1 ml dialytic medium was taken out for analysis and an equal volume of freshly prepared medium was supplemented. Absorbance of the dialytic medium at 270 nm was recorded by an UV–vis spectroscopy. The released amount of CH was quantified by referring to a calibration curve recorded from known amounts of CH at the same condition.

RESULTS AND DISCUSSION

Fabrication and properties of chitosan nanoparticle

The chitosan molecules are gelled when they encounter TPP molecules via electrostatic interaction.\(^{[10]}\) Firstly, the zone of particle formation is investigated. Chitosan was dissolved in 1.5% acetic acid solution to form a 10 mg/ml solution, which was diluted with water to various concentrations: 1.0, 2.0, 3.0, 4.0, and 5.0 mg/ml. The pH value of these solutions was adjusted to 4.8. TPP was dissolved in water at various concentrations: 0.50, 0.75, 1.0, 1.25, 1.50, 1.75, 2.0, and 2.5 mg/ml. Chitosan nanoparticles were prepared by the addition of 14 ml TPP solution into 35 ml chitosan solution at room temperature under mechanical agitation. By visual observation, the systems were classified as clear solution (\(\times\)), opalescent suspension (\(\checkmark\)), and aggregates (\(\#\)). The results are summarized in Table 1. A clear solution was observed when both the chitosan and TPP concentrations were too small, whereas aggregates were formed spontaneously when they were too large. The zone of the opalescent suspension, which should represent a suspension of colloidal particles, was found when the chitosan and the TPP concentrations were appropriate. Illuminating that formation of the nanoparticles is only possible for some specific concentrations of chitosan and TPP. Confirmed by DLS measurement and microscopy observation, the chitosan nanoparticles with a size of ~300 nm were obtained and well dispersed in the solution when the concentration of chitosan and TPP was 2.0 and 1 mg/ml, respectively.

Figure 1a shows that the number average size of the chitosan nanoparticles was measured as 321 nm by DLS, which is consistent with the TEM observation (Fig. 1c, d). No severe agglomeration of the particles was observed. A magnified TEM image (Fig. 1d) illustrates that the chitosan nanoparticles had a core–shell structure, i.e. with denser skirts and looser interiors. After loading with CH, the particle size increased slightly to 337 nm (Fig. 1b) with a homogeneous structure (Fig. 1e, f), suggesting that the drug was surely loaded.

For the next study, the chitosan concentration was fixed at 2 mg/ml with a pH value of 4.8. Fig. 2 shows that both the particle size (Fig. 2a) and zeta potential (Fig. 2b) decreased along with the increase of TPP concentration. This alteration tendency is consistent with the results of Gan et al.,\(^{[18]}\) but is the opposite

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**Table 1. Conditions for formation of the chitosan nanoparticles**

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<tr>
<th>CS (mg/ml)</th>
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×, clear solution; √, opalescent suspension; ⊥, aggregates.

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Figure 1. (a,b) Size distribution and (c–f) TEM images of (a,c,d) chitosan nanoparticles and (b,e,f) CH loaded chitosan nanoparticles, respectively. The chitosan and TPP concentrations were 2 and 1 mg/ml, respectively. This figure is available in color online at www.interscience.wiley.com/journal/pat.
to the results of Pan.\cite{22} We think the decrease is more acceptable, since at higher TPP concentration, the cross-linking degree of the chitosan nanoparticles is also higher. This will result in a more compact particle structure. Moreover, the neutralization degree of the charged amino groups is simultaneously improved, leading to smaller net charge in the particles as shown in Fig. 2b. Due to the compact structure and weakened charge repulsion, the particles prepared at higher TPP concentration a have smaller size.

At a fixed ratio of CS to TPP concentration, the particle size increased along with the increase of CS and TPP concentration (c) (Fig. 2c). At a given spatial interaction distance, the particle size should be roughly increased by a factor of $c^{1/3}$. Apparently the particle size shown in Fig. 2c does not follow this law. The reason can be both the change of spatial interaction distance and the particle inner structure, e.g. the compactness of the molecules.

As the association is driven by ionic interaction, and the charging degree of both chitosan and TPP is influenced by pH value, a pH variation during the fabrication is expected to influence the formulation and structure of the chitosan nanoparticles. Figure 3a shows that the nanoparticle size increased rapidly until pH 3.5, and then decreased slowly until pH 5.5, the measured pH value so far. A similar alteration tendency for the zeta potential was recorded with a slightly

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**Figure 2.** Influence of TPP concentration on: (a) the particle size, (b) zeta potential, and (c) chitosan concentration on the particle size at a fixed CS to TPP mass ratio of 4:1. The error bar indicates the standard deviation averaged from five measurements unless specifically indicated.

**Figure 3.** Influence of chitosan solution pH value on: (a) the particle size and (b) zeta potential.

**Figure 4.** Influence of particle suspension pH value on: (a) the size and (b) zeta potential.
higher transition pH value (pH 4) (Fig. 3b). This alteration behavior is dependent on charging properties and the interplay between the chitosan and the TPP molecules. At a critical low pH, most of the amino groups of chitosan are protonated, enabling the chitosan molecule with an extension confirmation due to strong charge repulsion. The TPP molecule is also protonated, leading to lower charging density of the molecule. In this case, the chitosan molecules cannot be sufficiently cross-linked by TPP to form stable particles. Along with increase of the pH value, the deprotonation degree of TPP is increased gradually, while the protonation degree of chitosan is less influenced when the pH value is below 5. At a proper pH such as pH 3.5, the charge interaction between these two molecules becomes strong enough, thus stable chitosan nanoparticles are obtained with a relatively larger size. At a still higher pH value, the charging degree of TPP molecule is enhanced again, which neutralizes the chitosan to a greater extent. Consequently, the particles shrink their size again.

Besides the fabrication process, the pH variation also influences the size of the formed nanoparticles as shown in Fig. 4a. The same decreasing tendency of the particle size was found exactly when the pH value was increased from 4.5 to 6.0. Meanwhile, the zeta potential was decreased too (Fig. 4b). However, the zeta potential showed an increasing regime when the pH value was increased from 1 to 3.5, the same phenomenon as shown in Fig. 3b. If considering only the interaction of chitosan and TPP, the zeta potential should be larger or at least should remain constant at low pH. The reason for this abnormal low value at low pH is not very clear so far. Nevertheless, this phenomenon implies that the positive surface charge of the chitosan particles is shielding, which can be a result of reorganization of the molecule structure, or/and adsorption of other negative charged ions at low pH. Of course, ionic strength effect at a critical low pH can not be excluded.

Figure 5. Influence of NaCl concentration on: (a) the particle size and (b) zeta potential.

Figure 6. Alteration of average size of chitosan nanoparticles with: (a) different TPP concentration and (b) at different pH during a storage time of 3–85 days at 4°C.

Figure 7. Cytoviability of fibroblasts measured by MTT assay as a function of culture time. Blank control was made by culture medium. Three parallel samples were analysed and data were expressed as mean ± standard deviation.
Stability and biocompatibility of the nanoparticles

For pharmaceutical applications, the storage stability and biocompatibility of the nanoparticles is a great concern. It is known that tiny particles are inclined to agglomerate with each other to reduce the surface area, and hence to reduce the free surface energy. Figure 6 presents the sizes of the nanoparticles prepared with different TPP concentration (Fig. 6a) and at different pH values (Fig. 6b) during a storage time of 3–85 days. Here the storage temperature was kept constant at 4 °C. All the particles were rather stable with neglectable size fluctuation within 15 days, and then some of them showed size increment at day 85 by a factor of <130%. Comparatively, the particles fabricated with a TPP concentration of 1.0–1.25 mg/ml at pH ~5.0 have the best storage stability. It is worth mentioning that the nanoparticles can spontaneously precipitate after being store undisturbed for a couple of days. But they are very easily re-dispersed by gentle shaking.

To assess the biocompatibility of the chitosan nanoparticles, in vitro culture of human fibroblasts was performed. Figure 7 compares the viability of fibroblasts as a function of time. No difference on the cytoviability was found between the cells cultured in the medium containing chitosan nanoparticles and the cells cultured in the control medium at all the culture time, demonstrating that these ionically cross-linked chitosan nanoparticles do not have any cytotoxicity.

CH loading and release

Many factors influence the loading and release performance of chitosan nanoparticles. As shown in the previous results, pH of the solution has a greater impact on the particle size and surface charge. Therefore, only the solution pH was varied here to explore its influence on the drug loading. Due to the difficulty of direct analysis of the nanoparticles, here the drug concentration in the supernatant was determined. A higher supernatant concentration implies a lower loading efficiency in the nanoparticles. Figure 8a shows that the CH concentration in the supernatant decreased with the increase of solution pH, thus the drug loading efficiency was increased. Although the particle shrinkage at a higher pH may be more or less unfavorable to the diffusion of CH, the major reason should be the stronger charge interaction between the CH and chitosan molecules. CH has a carboxylic group in its molecule, which is gradually deprotonated at higher pH and can combine with positively charged chitosan.

Considering the practical applications, the CH release from the chitosan nanoparticles was performed in pure water and in PBS (Fig. 8b). A burst release of the CH from both media was observed at the initial stage. Release of CH in pure water reached equilibrium after 2.5 hr later, while in PBS the release lasted at least for 10 hr. The total released amount of CH in PBS is larger too. This is quite normal since the ions in PBS can screen the charge interaction between both chitosan/TPP and chitosan/CH. Consequently, more CH can be released in PBS medium.

Conclusions

Chitosan nanoparticles are fabricated and their properties are manipulated and characterized in this work. Formulation of chitosan nanoparticles can be achieved at suitable concentrations zones of CS and TPP. Chitosan nanoparticles with a size of ~300 nm can be obtained when the concentration of chitosan and TPP was set as 2. and 1 mg/ml, respectively at pH 4.8. The pure chitosan nanoparticles and CH loaded ones have a core–shell and a filled structure, respectively. At a fixed chitosan concentration, a higher TPP concentration results in smaller particles. When the mass ratio of chitosan to TPP is fixed, the particle size increases along with the reactant concentration. Influence of solution pH is complicated. The particle size and zeta potential increase along with the pH increase below pH 3.5, but decrease again above pH 3.5. The nanoparticles show good storage stability at 4 °C, with no apparent agglomeration and severe size increase until 85 days. The nanoparticles have no significant influence on the fibroblast viability, demonstrating their good biocompatibility. The applicability of the chitosan nanoparticles as drug carriers is demonstrated by loading and release of a model drug, ciprofloxacin (CH). Indeed, the technique
described here is not restricted to CH, but can also be extended to other types of drugs, proteins, enzymes, and growth factors for different applications.

REFERENCES