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Effect of molecular structure of chitosan on protein delivery properties of chitosan nanoparticles

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Abstract

Chitosan nanoparticles (CS NP) with various formations were produced based on ionic gelation process of tripolyphosphate (TPP) and chitosan. They were examined with diameter 20–200 nm and spherical shape using TEM. FTIR confirmed tripolyphosphoric groups of TPP linked with ammonium groups of chitosan in the nanoparticles. Factors affecting delivery properties of bovine serum albumin (BSA) as model protein have been tested, they included molecular weight (Mw) and deacetylation degree (DD) of chitosan, the concentration of chitosan and initial BSA, and the presence of polyethylene glycol (PEG) in encapsulation medium. Increasing Mws of chitosan from 10 to 210 kDa, BSA encapsulation efficiency was enhanced about two times, BSA total release in PBS (phosphate buffer saline) pH 7.4 in 8 days was reduced from 73.9 to 17.6%. Increasing DD from 75.5 to 92% promoted slightly the encapsulation efficiency and decelerated the release rate. The encapsulation efficiency was highly decreased by increase of initial BSA and chitosan concentration; higher loading capacity of BSA speeded the BSA release from the nanoparticles. Adding PEG hindered the BSA encapsulation and accelerated the release rate.

Keywords: Chitosan; Bovine serum albumin (BSA); Nanoparticles

1. Introduction

The hydrophilic nanoparticles have received much attention to deliver therapeutic peptide, protein, antigen, oligonucleotide and gene by intravenous, oral, and mucosal administration (Janes et al., 2001a). The information has emphasized the importance of size, and revealed the advantages of nanoparticles over the microspheres (Meclean et al., 1998), it has been observed that the number of nanoparticles that cross the epithelium is greater than the number of microspheres. Chitosan is a biodegradable and bioadhesive polysaccharide. It has been shown that chitosan is non-toxic and soft tissue compatible in a range of toxicity tests (Aspden et al., 1997). It has been widely used in pharmaceutical research and in industry as a carrier for drug delivery and as biomedical material (Mao et al., 2001). Chitosan was selected for nanoparticles because of its recognized mucoadhesivity and ability to enhance

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the penetration of large molecules across mucosal surface (Illum, 1998).

More recently, researches have attempted to study chitosan nanoparticles as follows: preparation, modification, properties of loading various drugs and their physiological characters, such as chitosan nanoparticles coated PLGA (Vila et al., 2002) and PEO-PPO (Calvo et al., 1997), nanoparticles loaded insulin (Fernandez-Urrusuno et al., 1999), DNA (Mao et al., 2001) and anticancer drug doxorubicin (Janes et al., 2001b). But some important factors affecting drug properties have not always been investigated, such as basic molecular parameters of chitosan, molecular weight (Mw) and deacetylation degree (DD), were seldom evaluated in protein delivery system of nanoparticles. Janes stated the Mws of chitosan might have a role in the protein or peptide release in the review about chitosan nanoparticles as delivery systems for macromolecules (Janes et al., 2001a), but the relative paper has not been reported. The introduction of a second ingredient may increase their versatility in terms of the encapsulation and delivery of proteins and their susceptibility to interact with biological surface. Polyethylene glycol (PEG) coated nanoparticles have been found to be important potential therapeutic application for controlled release of drugs and site-specific drug delivery (Quellec et al., 1998). Few studies have attempted to investigate chitosan nanoparticles coated with PEG. Therefore, we investigate a series of factors affecting delivery properties, the influence of Mw and DD of chitosan, concentration of chitosan and initial bovine serum albumin (BSA), and PEG introduction are all evaluated.

The purpose of the current study was to examine the influence of a number of factors on the encapsulation of a model protein BSA and release properties during incubation in phosphate buffer saline (PBS) of pH 7.4, and investigate the physicochemical structure of nanoparticles by FTIR and TEM. A series of chitosan nanoparticles with various molecular parameters were prepared, effect of Mw and DD of chitosan on protein delivery was studied systematically. Effect of initial BSA and chitosan concentration and PEG presence was also evaluated. Thus, we can modulate their encapsulation capability and release rate by adjusting the molecular and formation parameters.

2. Material and method

2.1. Material

Chitosan (CS) from a shrimp shell was purchased from Yuhuan Ocean Biochemical Co. (Zhejiang, China), DD was 92%, and Mw was 210 kDa. Chitosan of DD 92% with different Mws (115, 80, 48, 10 kDa) and chitosan of Mw 210 kDa with different DDs (75.2, 85.5, 92%) were prepared according the reference (Qin et al., 2002; George and Frances, 2001). The Mws were measured by a gel permeation chromatography (GPC), the DDs were determined by elemental analysis. BSA with Mw 68 kDa, PEG with Mw 20 kDa, and Coomassie brilliant blue G250 were purchased from Sigma Chemical Co. (USA). All other chemicals were of reagent grade.

2.2. Preparation of chitosan nanoparticles and BSA loaded nanoparticles

Chitosan with various Mw and DD was dissolved in acetic aqueous solution at different concentration. The concentration of acetic acid in aqueous solution was, in all case, 0.5 times higher than that of chitosan. Then, tripolyphosphate (TPP) was dissolved in distilled water at 0.7 or 1.0 mg/ml. Finally, 2 ml of TPP solution was added to 5 ml of the chitosan solution, opalescent suspension was formed spontaneously under magnetic stirring at room temperature, and was further examined as nanoparticles.

In some preparations PEG of 10 mg/ml was dissolved in the chitosan solution before adding TPP solution. The BSA loading nanoparticles were formed upon incorporation of TPP solution to chitosan solution containing various concentration of BSA. The detailed formation conditions were shown in the corresponding legends and figures.

The factors tested included BSA initial concentration (0.1, 0.2, 1, 1.5, 2 mg/ml), Mws of chitosan (210, 115, 80, 48, 10 kDa), DDs of chitosan (75.2,

85.5, 92%), and chitosan concentration (1, 1.5, 2, 2.5, 3 mg/ml). When we evaluated one factor, we only altered its own parameters, and kept the parameters of other factors constant. Without specific depiction, the parameters of chitosan were as follows: concentration 2 mg/ml, DD 92% and Mw 210 kDa; initial concentration of BSA was 1.5 mg/ml; TPP concentration was 1 mg/ml.

2.3. Morphology and structure characterization of nanoparticles

The morphology and particles size measurements of the nanoparticles were performed by TEM-100CXII. Chitosan nanoparticles separated from suspension were dried by a freeze dryer, their FTIR were taken with KBr pellets on Perkin–Elmer Spectrum one FTIR.

2.4. Determination of BSA encapsulation efficiency of nanoparticles

Encapsulation efficiency and loading capacity of nanoparticles with the different formation were determined by ultra-centrifugation of samples at 20,000 × g and 15 °C for 30 min, the amount of free BSA was determined in clear supernatant by UV spectrophotometry at 280 nm using supernatant of non-loaded nanoparticles as basic correction. The BSA loading capacity (LC) of nanoparticles and the BSA encapsulation efficiency (AE) of the process were calculated from Eqs. (1) and (2) indicated below:

$$\mathrm{LC} = (A - B)/C \times 100 \quad (1)$$

$$\mathrm{AE} = (A - B)/A \times 100 \quad (2)$$

A, total amount of BSA; B, free amount of BSA; C, nanoparticles weight.

2.5. BSA release from the nanoparticles in vitro

The in vitro BSA release profiles of chitosan nanoparticles were determined as follows. The BSA loaded chitosan nanoparticles separated from 18 ml suspension were placed into test tubes with 6 ml of 0.2 mol/l PBS of pH 7.4, and incubated at 37 °C under stirring. At appropriate intervals samples were ultra-centrifuged, and 4 ml of the supernatant were replaced by fresh medium. The amount of BSA released from the nanoparticles was evaluated by modified Coomassie Brilliant Blue protein assay (Pierce, Inc, New York, NY, USA). The calibration curve was made using non-loaded BSA nanoparticles as correction.

3. Results and discussion

3.1. Physicochemical characterizations of nanoparticles

TEM of the nanoparticles and their surface morphology are shown in Fig. 1, they are about 20 nm in size and spherical in shape. Compared with chitosan nanoparticles, the surfaces of nanoparticles contained PEG exhibits fluffy. It has been previously reported that the incorporation of PEG in gel system is through intermolecular hydrogen bonding between the electro-positive amino hydrogen of chitosan and the electro negative oxygen atom of PEG, thus forming a CS/PEG semi-interpenetration network (Kim and Lee, 1995). So we suppose that PEG is attached to the nanoparticles surface.

FTIR spectra of chitosan nanoparticles and chitosan matrix are shown in Fig. 2. A band at 3434/cm has been previously attributed to $-NH_2$ and –OH group stretching vibration in chitosan matrix (Yu et al., 1999). In chitosan nanoparticles a shift from 3434 to 3399/cm is shown, and the peak of 3399/cm becomes wider, this indicates hydrogen bonding is enhanced (Yu et al., 1999). In nanoparticles the shoulder peak of 1644/cm disappears and a new sharp peak 1630/cm appears, and the 1602/cm peak of $-NH_2$ bending vibration shifts to 1534/cm. Knaul observed the similar result in the study of chitosan film treated with phosphate (NaH_2PO_4), and attributed it to the linkage between phosphoric and ammonium ion (Knaul et al., 1999). So we suppose that the tripolyphosphoric groups of TPP are linked with ammonium group of chitosan, the inter and intramolecular action are enhanced in chitosan nanoparticles.

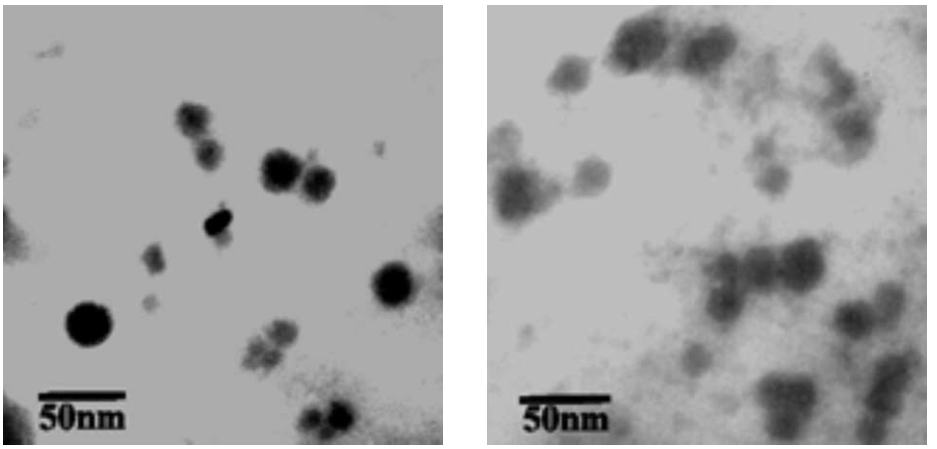

Fig. 1. TEM of CS (a) and CS/PEG (b) nanoparticles (Mw = 48 kDa, CS 1.5 mg/ml, TPP 0.7 mg/ml).

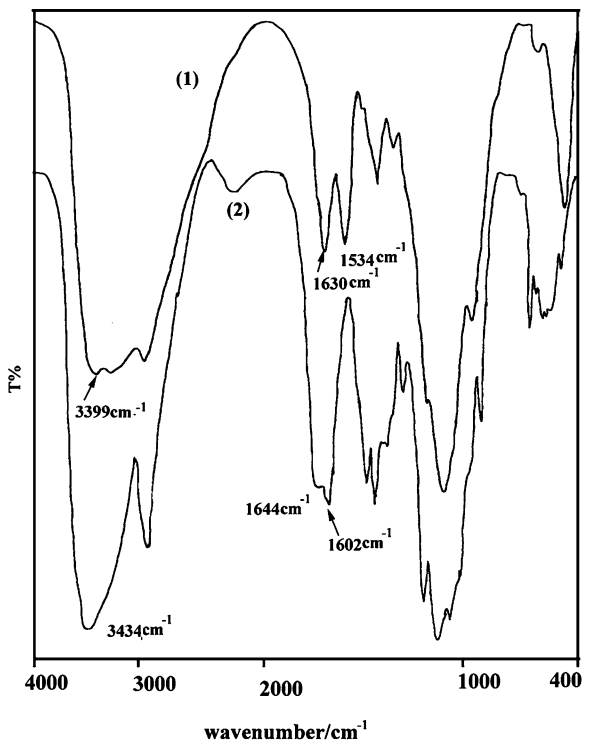

Fig. 2. FTIR of chitosan nanoparticles (1) and chitosan (2).

3.2. Encapsulation of BSA within nanoparticles

3.2.1. Effect of BSA concentration and PEG introduction

BSA encapsulation efficiency from 10 to 90% was significantly affected by the initial BSA concentration in Fig. 3, the lower the concentration, the higher the encapsulation efficiency. However, the protein loading was enhanced dramatically from 26 to 47% by increasing the initial BSA concentration from 0.2 to 2 mg/ml in Fig. 4. On the other hand, protein encapsulation efficiency of chitosan nanoparticles was decreased with the addition of PEG in chitosan solution, their loading capacity was also decreased. As reported previously (Kim and Lee, 1995), inter molecular hydrogen bonding can be formed between the electro negative oxygen atom of PEG and the amino groups of chitosan in gel system. The acid group of BSA and oxygen atom of PEG may compete in their interaction with chitosan amino groups, so the possibilities of an interaction between the BSA and the chitosan are reduced, the entanglement of PEG chains with the chitosan molecules hinders the encapsulation of BSA into the nanoparticles.

3.2.2. Effect of chitosan concentration

When TPP concentration was 1 mg/ml, decreasing chitosan concentration down to 0.5 mg/ml,

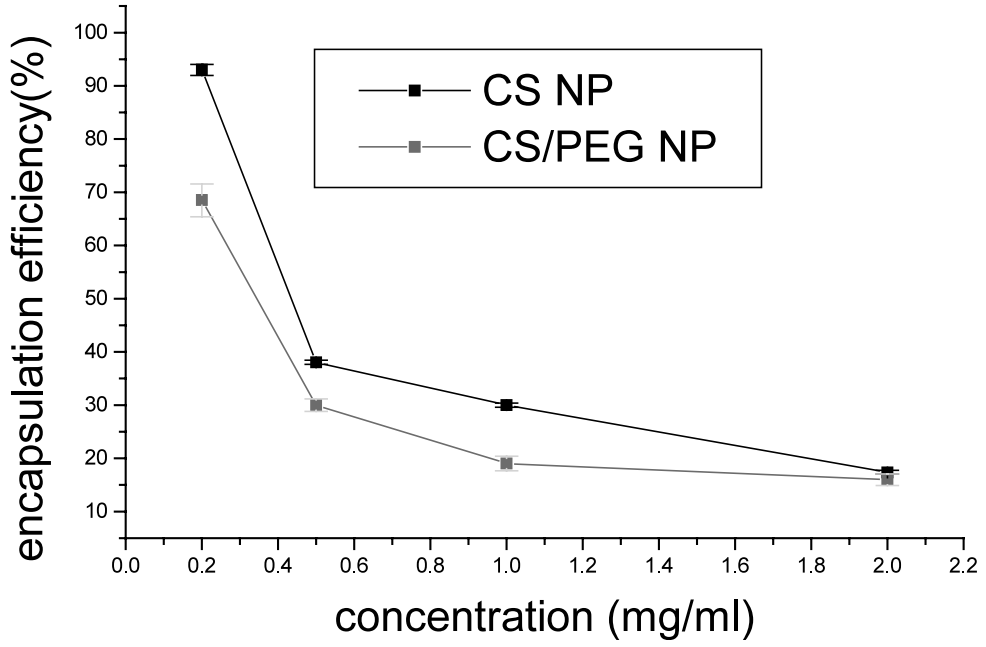

Fig. 3. The influence of BSA initial concentration on encapsulation efficiency (CS 1.5 mg/ml, TPP 0.7 mg/ml, $n = 4$).

aggregates with large diameter were formed; increasing chitosan concentration up to 4 mg/ml made encapsulation extremely difficult. Formation of nanoparticles is only possible for some specific concentration of chitosan and TPP. As for gelation between TPP solution of 1 mg/ml and chitosan solution of 1–3 mg/ml, we usually observed that some opalescent suspension was

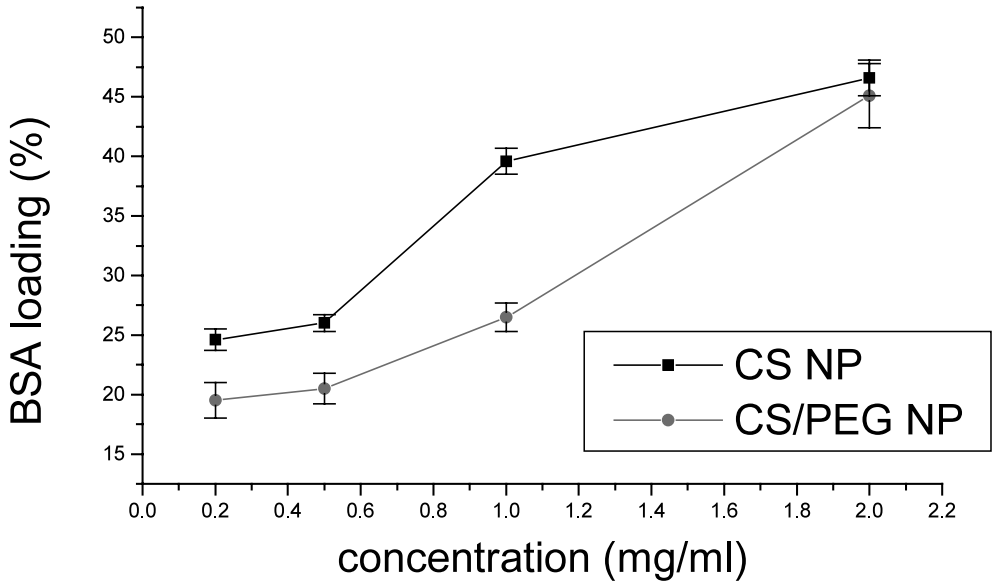

Fig. 4. The influence of BSA initial concentration on loading capacity (CS 1.5 mg/ml, TPP 0.7 mg/ml, $n = 4$).

formed, which was examined as nanoparticles. Adding TPP solution of 1 mg/ml to chitosan solution of 4 mg/ml, firstly we observed some opalescent suspension, and then disappeared immediately, the nanoparticles formation was extremely difficult. Fig. 5 shows that increasing the chitosan concentration decreased encapsulation efficiency of BSA. Vandenberg stated that the highly viscous nature of the gelation medium hinders encapsulation of BSA in the study of chitosan-alginate microspheres (Vandenberg et al., 2001). Relatively lower adhesivity of chitosan with lower concentration promotes encapsulation of BSA and gelation between chitosan and TPP.

3.2.3. *Effect of DD and Mw*

Fig. 6 shows that as DD of chitosan increased, the encapsulation efficiency increased. Here Mw of each sample is 210 kDa, chitosan with higher DD contains more functional groups, which can complex with the acid groups of BSA and gelate with the tripolyphosphoric groups (Sabnis and Block, 2000), so the encapsulation efficiency of BSA increases correspondingly.

As shown in Fig. 7, as the Mw of chitosan increased, the encapsulation efficiency of BSA was increased directly. There is ample evidence attesting to the effect of Mw of chitosan on its complexation behavior. Wu et al. found that chitosan effectiveness in coagulating solids and proteins was inversely proportional to its Mw (Sabnis and Block, 2000), they stated that the depolymerization affords formation of chitosan having a greater number of amino groups available for interactions with the anionic actives. But Flory stated that, in general, the intrinsic reactivity of all functional groups on a polymer remains the same (Flory, 1953). We agree with Flory, chitosan with various Mw but the same DD have the same functional groups. Compared with molecular chains of BSA with 68 kDa, the chains of chitosan with Mw down to 48 kDa are relatively short, they possibly make entrapment of BSA difficult, so their encapsulation efficiency is much lower. The encapsulation capacity of Mws 210 and 115 kDa is much greater, this is possibly attributed to their longer chains of molecular, which can entrap greater amount of BSA when gelate with TPP.

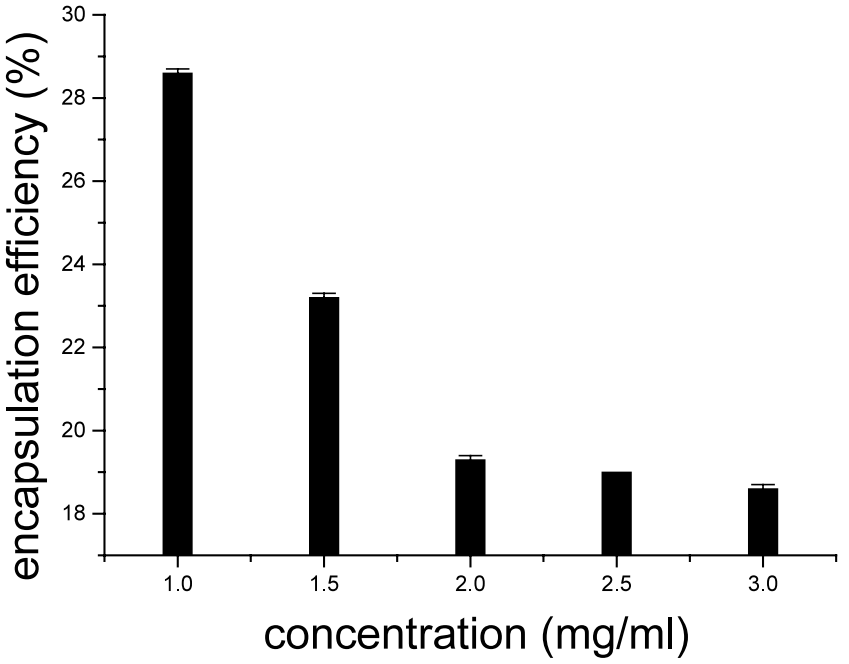

Fig. 5. The influence of chitosan concentration on BSA encapsulation efficiency (TPP 1 mg/ml, $n = 4$).

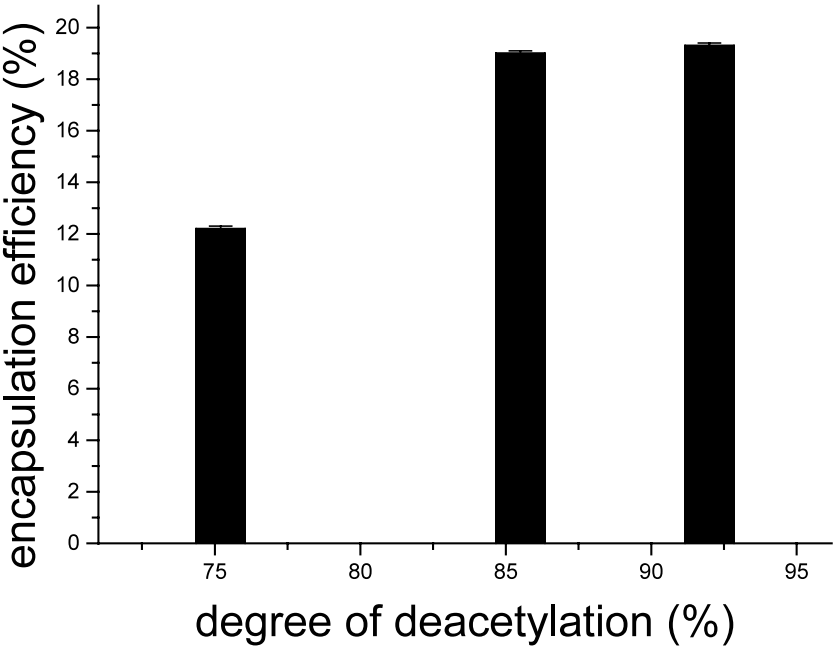

Fig. 6. BSA encapsulation efficiency of CS NP with different DD (Mw 210 kDa, $n = 4$).

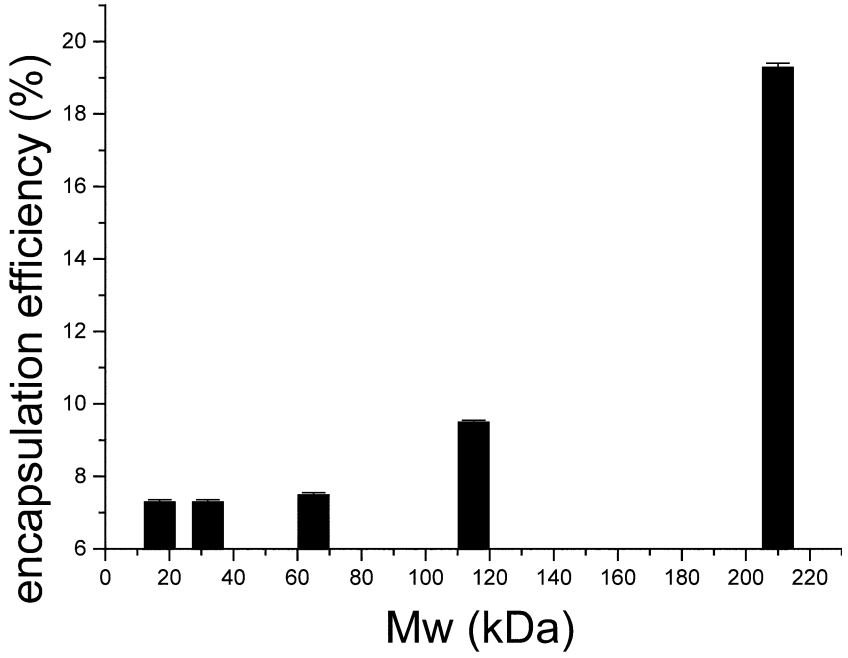

Fig. 7. BSA encapsulation efficiency of CS NP with various Mw of chitosan (DD 92%, $n = 4$).

3.3. In vitro release

The BSA in vitro release behavior of chitosan is shown from Figs. 8–11. All release profiles of the nanoparticles are similar, and exhibit a small burst release about 10% in the first 12 h, and then slow release at constant but a different rate. The results reveal that there are possibilities to modulate the release rate of BSA by adjusting the concentration of BSA and chitosan, or molecular parameters of chitosan.

Some reports about microspheres revealed that the release involves two different mechanisms, that is, diffusion of protein molecules and degradation of polymer matrix. The burst release of protein is associated with those protein molecules dispersing close to the microspheres surface, which easily diffuse out in the initial incubation time (Zhou et al., 2001). However, it is uncertain that this hypothesis is suitable to BSA release from nanoparticles, the chain of BSA molecular is much longer than the size of nanoparticles, so it is difficult that BSA moleculars diffuse through the surface or pores of nanoparticles in a short time. The nanoparticles with huge specific surface area can adsorb BSA, so the first burst release is possibly due to this part of BSA desorbed from nanoparticles surface.

3.3.1. Effect of BSA loading and PEG presence

BSA loading capacity is an important factor, as for release property of microspheres, the release rate is usually drug concentration gradient driven, higher levels of loaded drug lead to a wider concentration gap between the polymeric microspheres and the release medium, and cause a higher diffusion rate. As shown in Fig. 8, BSA release rate was influenced by the amount of protein entrapped, the high loading capacity provided a fast release rate. Difference release rate between the nanoparticles with 26 and 47% of loading capacity is just attributed to BSA concentration gradient. PEG introduction hinders the BSA encapsulation as shown in Fig. 3, but accelerates BSA release rate as shown in Fig. 8. The entanglement of PEG chains with the chitosan molecules hinders the packed and rigid bonding between chitosan and BSA, so relatively loose structure of nanoparticles contained PEG results in a high rate of BSA release.

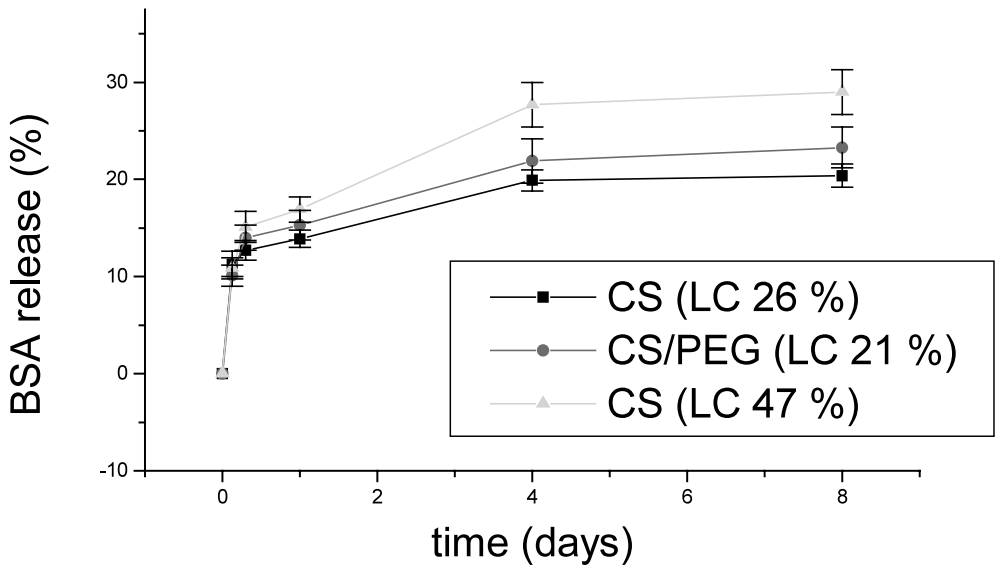

Fig. 8. The influence of loading capacity and PEG presence on BSA release behavior (CS 1.5 mg/ml, TPP 0.7 mg/ml, $n = 3$).

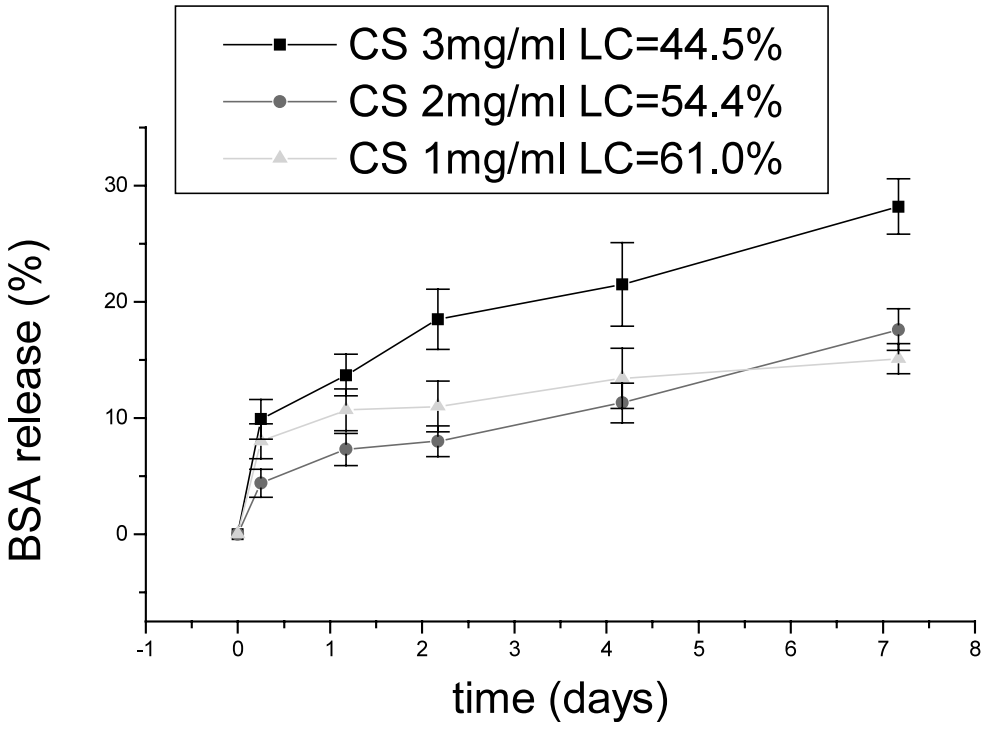

Fig. 9. The influence of CS concentration on BSA release behavior (Mw 210 kDa, DD 92%, $n = 3$).

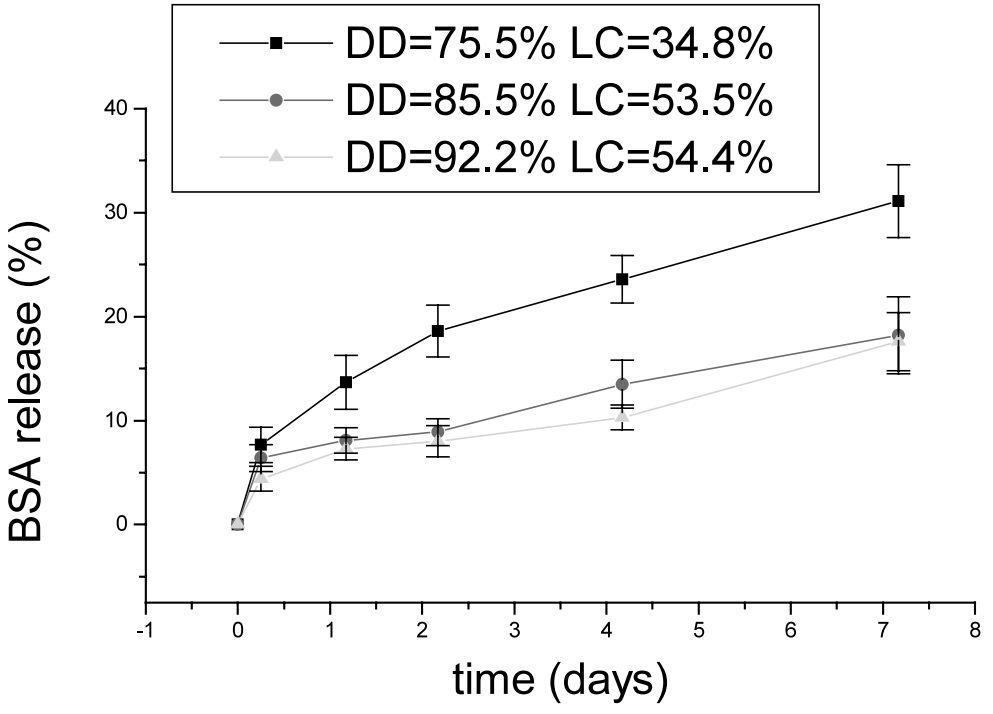

Fig. 10. The influence of DD of CS on BSA release behavior (Mw 210 kDa, $n = 3$).

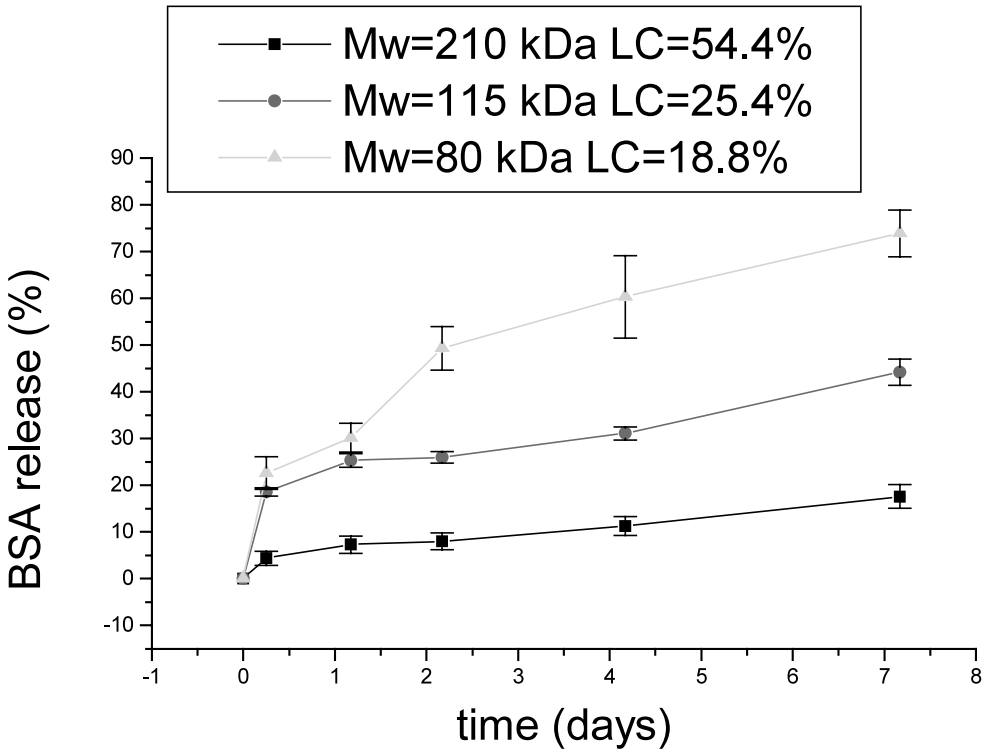

Fig. 11. The influence of Mw of CS on BSA release behavior (DD 92%, $n = 3$).

3.3.2. Effect of chitosan concentration

The highest concentration of 3 mg/ml provides the highest release rate, both of them do not show a direct relationship in Fig. 9. Some studies about alginate-chitosan microcapsules reported that a higher concentration of chitosan provides a more viscous medium, thus permitting a lower capacity for interaction with the alginate microcapsule, resulting in a thinner, more permeable membrane, and higher release rate (Daly and Knor, 1988). This is coincident with the result of encapsulation and release of different chitosan concentration. As for both chitosan concentration of 1 and 2 mg/ml, the factor of loading capacity of nanoparticles is also important for release rate. In the first 6 days, the release rate is drug concentration gradient driven, higher levels of loaded drug lead to initial higher diffusion rate, and causes the observed initial difference. However, 6 days later, as the amount of drug within the nanoparticles gradually decreases, the membrane permeability becomes the main factor controlling the release profile, so a higher concentration results in a more permeable membrane, and promotes BSA release.

3.3.3. Effect of DD and Mw

Higher DD of chitosan decreased BSA release rate, and influence of DD was a main factor and more important than that of loading capacity in Fig. 10. As revealed in analyses of FTIR of chitosan matrix and nanoparticles, strong gelation and hydrogen bonding are formed in the nanoparticles. Higher DD of chitosan with the same Mw provides more compact nanoparticles due to the greater number of ammonium groups of chitosan gelated with tripolyphosphoric groups, so lower permeability of nanoparticles surface results in slow release rate.

As shown in Fig. 11, as the Mw of chitosan increased, the release rate decreased. Contrary to the result in Fig. 8, a higher loading capacity showed a lower release rate, this indicates that the factor of Mw is more important than that of loading capacity. In a week BSA release percent of

nanoparticles with Mws of 210, 115 and 80 kDa were 17.6, 44.2 and 73.9%, respectively, the release of lower Mw is much faster. The influence of Mw of polymer on nanoparticles surface permeability has not been reported, the mechanism is not clear. With regard to microspheres, membrane permeability being related directly to Mw of polymer has been observed in some studies, using poly-L-lysine for membrane formation in alginate microcapsules, increased diffusion of BSA with increasing Mw has been reported (Goosen et al., 1985; King et al., 1987). However, Okhamafe and Goosen (1993) suggested that, theoretically, membrane permeability of coacervate microcapsules would be lower with increasing Mw due to increased chain packing and rigidity, as well as increased inter-chain bonding, this theory is helpful to interpret the results we observed.

4. Conclusion

In the present study, the physicochemical structure of chitosan nanoparticles is different from matrix chitosan, inter and intramolecular action is enhanced due to tripolyphosphoric groups of TPP gelation with ammonium groups of chitosan. Formation and molecular parameters of chitosan nanoparticles play an important role in protein delivery. Altering concentration of BSA from 0.2 to 2 mg/ml and chitosan from 3 to 1 mg/ml enhance significantly encapsulation capacity of BSA. Nanoparticles preparation from chitosan with various DDs from 75.5 to 92% and Mws from 10 to 210 kDa promotes encapsulation capacity and decreases the release rate. So we can modulate relative parameters to satisfy the needs of protein delivery.

Acknowledgements

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