QUATERNIZED CHITOSAN/ALGINATE NANOPARTICLES FOR PROTEIN DELIVERY

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Abstract

Quaternized chitosan (QCS)/alginate (AL) nanoparticles (QCS/AL) were successfully prepared in neutral environment for the oral delivery of protein. The physicochemical structure of the QCS/AL nanoparticles was characterized by IR spectroscopy and transmission electron microscopy (TEM). The diameter of the nanoparticles with a positive surface charge was about 200 nm. The load of bovine serum albumin (BSA) was affected by the concention and the molecular parameters, i.e. degree of substitution (DS) and weight-average molecular weight (Mw) of QCS, as well as the concentration of BSA. The release of BSA from nanoparticles was pH-dependent. Quick release was occurred in 0.1 M phosphate buffer solution (PBS, pH=7.4) while the release was slow in 0.1M HCl (pH=1.2). The DS and Mw of QCS play important roles in the relese of BSA in vitro. QCS with high Mw accelerated the release of BSA in acid while high DS retarded the release of BSA both in 0.1M HCl and in 0.1 M PBS.

Introduction

Significant advances in biotechnology and genetic research have resulted in the discovery of a large number of proteins and peptides which are very effective in disease treatment.[1,2] Normally, peptides and proteins are administered by the parenteral route, which has poor patients acceptance. Large amount of work have focused on protein delivery by the oral route.[3-5] However, the bioavailability of peptide after oral administration is normally low, due to insufficient stability and poor absorption pattern of protein in the gastrointestinal (GI) tract. One possibility to improve the gastrointestinal uptake of peptide drugs is to encapsulate them in colloidal nanoparticles which can protect peptide from degradation in the gastrointestinal tract and promote their transport into systemic circulation.[6,7] It has been found that delivery systems with mean diameters in the range of hundreds of nanometers have a greater ability to penetrate the epithelia when compared to the particles in the micrometer size range.[8,9]

Recently, nanoparticles made from natural biodegradable polymers to deliver drugs have raised great interests. Since synthetic biodegradable polymers such as (PGA), polylactide (PLA),[10] and their copolymers,[11,12] are not ideal carriers for hydrophilic drugs like peptides and protein because of their strong hydrophobic property. Moreover, the organic solvent involved in the preparation procedure may reduce the activity of the protein.

Alginates are natural, anionic polysaccharides consisting of a chain of (1-4)-linked β-D-mannuronic acid and α-Lguluronic acid in different arrangements and quantities of the uronic acid residues. Alginate has been reported to be mucoadhesive, biodegradable and biocompatible enabling numerous pharmaceutical and biomedical applications such as drug delivery systems and cell encapsulation.[13] Alginate micro and nanoparticles can be obtained easily by inducing the gelation with calcium chloride.[14,15] Such a property can be used to produce a pre-gel consisting of very small aggregates of gel particles, followed by the addition of an aqueous polycationic solution to make a polyelectrolyte complex coating.[16] Poly-L-lysine (PLL), a cationic natural polymer has been used to combine with alginate to prepare nanoparticles. However, PLL is toxic and immunogenic when injected. Recently, chitosan was chosen as an alternative cation polymer. Chitosan, a linear

polysaccharide consisting of glucosamine and N-acetylglucosamine units, is biocompatible, biodegradable and nontoxic merit in the application of peroral delivery of peptide.[17] However, chitosan is insoluble at neutral aqueous solution and only soluble in acidic solutions with pH values lower than 6.0. This interferes with the biomedical application of chitosan when some vulnerable peptides or DNAs loaded in chitosan matrix often dissolved in acid.

N-(2-hydroxyl) propyl-3-trimethyl ammonium chitosan chloride is a partially quaternized derivative of chitosan with improved solubility and enhanced positive charge intensity. It has previously been shown that quaternized chitosan (QCS) has excellent absorption enhancing effects across mucosal epithelia even in neutral environment.[18,19] Owing to the good water solubility of QCS, it is appealing to prepare peptide loaded QCS coated calcium-alginate nanoparticles in neutral water phase. In the paper, QCS/Alginate nanoparticle (QCS/AL) was obtained by ionotropic gelation of QCS with the calcium alginate pre-gel. This system may have some interesting features. First of all, the nanoparticles are obtained spontaneously under very mild conditions; Second, the particle size can be well controlled through adjusting experimental condition; What's more, the out-layer of QCS can not only endow nanoparticles positive surface charge, but also increase the contact time of the active ingredients with the epithelium and absorption via the paracellular transport pathway through the tight junctions.[20] Also it noted that the nanoparticles have pH-dependent release behavior, which can protect drug loss in acid environment as well as control release in intestine tract.

The influence of the molecular factors of the cationic polymers on the formation and release properties of the alginate and chitosan nanoparticles has scarcely been investigated. Here, we look into the effect of Mw and DS of QCS on the nanoparticle formation and the delivery of BSA. Series of QCS with various average-weight molecule weight (Mw) and degree of substitution (DS) were synthesized and QCS/Alginate nanoparticles were prepared. The physicochemical structure of nanoparticles was investigated by FT-IR and TEM. The influence of Mw and DS of QCS, concentration of alginate and QCS on the encapsulation and release properties of BSA was also evaluated. This was done in order to optimize encapsulation system as an oral delivery system and modulate release rate by adjusting the molecular and formation parameters.

Experimental Section

Materials

Sodium alginate was purchased from Shanghai Chemical Reagent Co. (Shanghai, China). The intrinsic viscosity at 25 ^{o}C was 550 mL/g as measured by a capillary viscosimeter and the M/G ratio was 1.3 as measured by ^{1}H-NMR. Chitosan was supplied by Yuhuan Ocean Biochemistry Co., Ltd. (Zhejiang, China) and the deacetylation degree (DS) was 0.93. BSA with Mw 68 kDa was purchased from Sigma Chemical Co. (USA). All other chemicals were of analytical reagent grade.

Preparation of QCS with various Mw and Ds

Quaternized chitosan was prepared based on our previous work.[21] QCS with different Mw was obtained by H_2O_2 degradation. QCS (5.0 g) was dissolved in 100 mL distilled water and 1 mL H_2O_2 (30%, v/v) was added and stirred at 50 ^{o}C, samples were withdrawn in 0, 1, 2, 4 h and concentrated under reduced pressure. Mws of QCS were measured to be 230, 130, 40, 12 kDa by GPC analysis connected with a refractive index detector (Thermoquest, USA) and the standards used to calibrate the column were TOSOH pullulan. It has been proved that DS was affected by the ratio of chitosan to EPTMAC. [21] In order to obtain quaternary chitosan with different DS, the mole ratio of chitosan/EPTMAC was changed as 1/4, 1/5 and 1/6. The DS was determined by potentiometry to be 0.61, 0.82, and 0.95, respectively. [22]

Preparation of QCS-Alginate nanoparticles

QCS-Alginate nanoparticles were prepared using a method modified from Rajaonarivony et al.[14] Aqueous calcium chloride (2 mL of 0.5 mg/mL) was added dropwise to 6.0 mL of aqueous sodium alginate (1.0 mg/mL) under stirring. And then 1 mL of an aqueous QCS solution (1.0 mg/mL) with various Mw and DS was added to the calcium alginate pre-gel and stirred for 30 min. The resultant

opalescent emulsion with a pH of 6.85±0.1 was equilibrated overnight to allow nanoparticles to form and have uniform particle size. These nanoparticles were isolated by ultracentrifugation at 15,000 g for 30 min at 4 °C. The BSA-loaded nanoparticles were formed in the same way by adding $CaCl_2$ solution to alginate solution containing 1.0 mg/mL BSA. The amount of encapsulated protein was determined as the difference between the initial amount of protein and that remaining in the supernatant. The amount of free BSA in the clear supernatant was measured by UV spectrophotometry at 595 nm using a Bradfold protein assay.[23]

Morphology and structure characterization of the nanoparticles

Transmission electron microscopy (TEM, 100 CX II, Japan) was used to observe the morphology of the QCS/AL nanoparticles. Samples were stained with phosphotungstic acid solution (2%, w/v) and dried on copper grill at room temperature. The mean size and zeta-potential measurements of the QCS/AL were determined with a Zetasizer 3000HS (Malvern, UK). The size measurements were done with a wavelength of 532.0 nm at 25 °C with an angle detection of 90°. Each sample was measured 3 times repeatedly. For zeta-potential measurements, samples were appropriately diluted with 0.001 M NaCl solution in order to maintain a constant ionic strength and tested at automatic mode. FT-IR spectra were measured by a 170SX Fourier transform-infrared spectrometer (Nicolet, USA). The nanoparticles were lyophilized and mixed with KBr to press a plate for measurement.

In vitro release studies

The in vitro release profiles of BSA from QCS/AL nanoparticles were determined as follows: the BSA loaded QCS/AL separated from 9 mL suspension was placed into test tubes with 6 mL of 0.1 mol/L HCl or 0.1 mol/L PBS of pH 7.4, and incubated at 37 °C under stirring at 100 rpm. At appropriate intervals samples were ultra-centrifuged, and 1 mL of the supernatant was replaced by fresh medium. The amount of BSA released from the nanoparticles was evaluated by Coomassie Brilliant Blue protein assay as described earlier. The calibration curve was made using nonloaded BSA nanoparticles as correction. All release tests were run in triplicate and the mean value was reported.

Results and Discussion

Preparation of QCS/AL nanoparticles

Table 1 Effect of the concentration of QCS (Mw 230 kDa, DS 0.82) on the size of nanoparticles.

Samples	Na-alginate/CaCl2/QCS (w/w/w)	Status	Mean size (nm)
1	6:1:0.5	Solution	—
2	6:1:1	Light opalescent	280.5±16.2
3	6:1:1.5	Light opalescent	494.8±23.3
4	6:1:2	Opalescent	528.6±18.1
5	6:1:3	Opalescent	686.9±13.2
6	6:1:4	Opalescent	1045.2±32.5
7	6:1:5	Suspension	1151.9±26.4
8	6:1:6	Aggregation	—

As a matter of fact, calcium ions react with guluronic acid units on alginate to form an “egg-box” structure. It is proposed that nanoparticles can be formed by enveloping the negatively charged calcium alginate complex in pre-gel state with cationic polymer, and the pre-gel state is essential to enable the ionic interactions between alginate, calcium and cationic polymer to form nanoparticle. It was reported that a calcium chloride to sodium alginate mass ratio less than 0.2 was necessary to maintain the pre-gel state for the preparation of nanoparticles.[16] In this study, the ratio of sodium alginate to $CaCl_2$ was maintained constant at 6:1 (w/w) to produce pre-gels. To identify the effective concentration range of the polymeric cation on nanoparticles formation, different

concentration of QCS was added to the pre-gel (Table 1). Higher amount of QCS would result in the larger resulting particle size. The mean size spanned a broad range from 275 to 1150 nm when the QCS concentration increased from 1.0 to 2.0%. In the case of sample 2, particle size measurement by dynamic light scattering (DLS) and the observance of bluish Tyndall effect confirmed the formation of nanoparticles. This alginate/$CaCl_2$/QCS ratio was fixed during following preparation assay.

Effect of Mw and DS of QCS on the size of nanoparticles

It is anticipated that Mw and DS of QCS might play a role in the entrapment and release of BSA. Table 2 shows the mean diameter of QCS-alginate nanoparticles prepared from QCS with various Mw and DS. With the increased Mw of QCS, the diameter tested by DLS of the nanoparticles increased. This was expected that longer molecular chains of QCS with larger Mw entangled with negatively charged calcium alginate complex through ionic interactions, giving rise to bigger nanoparticles. However, The DS of QCS had no obvious effect on the nanoparticle diameter. These results indicated that the size of nanoparticles was mainly affected by the concentration and the Mw of QCS.

Table 2 Effects of the Mw and DS of QCS on the size of nanoparticles.

Ds	Mw (kDa)	Na-alginate/$CaCl_2$/QCS (w/w/w)	Mean size (nm)
c.a. 0.82	230		280.5±16.2
	130		253.5±12.3
	40		192.1±9.3
	15	6:1:1	151.2±11.2
0.95			256.6±14.2
0.82	c.a. 230		280.5±16.2
0.61			271.6±13.6

Characterization of the nanoparticles

Figure 1 shows the morphological characteristic of the typical QCS/AL. They are spherical in shape and the size is about 200 nm. However, the nanoparticles don't show a smooth surface but a fluffy appearance. The zeta potential of QCS/AL was 9.8±0.6 mV as measured, so we suppose that QCS are surrounded the outlayer of the nanoparticles.

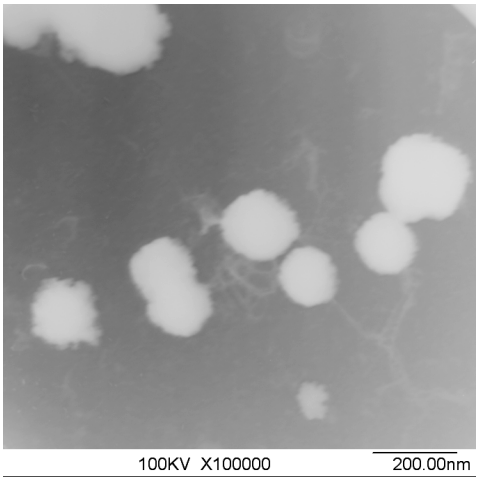

Figure 1: TEM of QCS/AL nanoparticles (the ratio of QCS:AL:$CaCl_2$ was 6:1:1 (w/w/w) with QCS Mw=40kDa, DS=0.82).

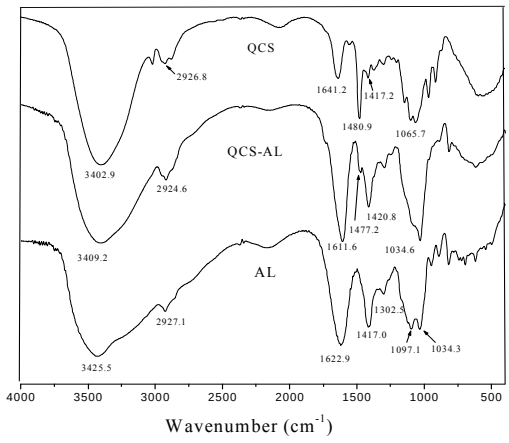

Figure 2. FTIR of QCS, AL and QCS/AL nanoparticles.

FT-IR was adopted to characterize the potential interactions in the nanoparticles. FT-IR spectras of alginate, QCS and QCS/AL are shown in Figure 2. In the spectra of QCS, The broad band at 3400

cm^{-1} corresponded to the amine and hydroxyl groups; the peak near 3000 cm^{-1} was caused by C-H stretching and the strong absorption peak appeared at 1480 cm^{-1} was attributed to the methyl groups of the ammonium. The IR spectrum is consistent with the reported spectra, [24] suggesting the successful chemical modification of chitosan. The bands around 1030 cm^{-1} (C–O–C stretching), and 950 cm^{-1} (C–O stretching) present in the IR spectrum of sodium alginate are attributed to its saccharide structure. In addition, The bands at 1620 and 1416 cm^{-1} are assigned to asymmetric and symmetric stretching peaks of carboxylate salt groups.[25] These results indicate that the carboxylic groups of alginate have been dissociated into $–COO^-$ groups that can complex with trimethylammonium groups of QCS through electrostatic interactions to form the polyelectrolyte complex. So in the IR spectrum of nanoparticles we can observe the asymmetrical stretching of $–COO^-$ groups shifted to 1611 cm^{-1} and the symmetrical stretching of $–COO^-$ groups shifted to 1420 cm^{-1}. In addition, the peak at 1480 cm^{-1} becomes a shoulder peak and the intensity decreased sharply, which means the content of QCS in the nanoparticles is relatively low.

Entrapment of BSA in nanoparticles

The pH value at which we prepared nanoparticles was 6.85±0.1. Taking into account that BSA has an isoelectric point of 4.8, the negatively charged BSA tends to interact with QCS driving by the electrostatic force in the neutral environment. The influence of Mw and DS of QCS on the load of BSA was studied. A comparison of the encapsulation efficiencies of BSA for QCS/AL prepared from QCS with different Mw is shown in Figure 3. It can be seen that the encapsulation capacity of QCS/AL with Mw 230 and 130 kDa is much higher than that of QCS with Mw 15 kDa. This is possibly attributed to their longer molecular chains, which can entangle with the alginate core through electrostatic and hydrophobic interactions. At the same time, higher Mw of QCS gave rise to larger nanopshere, as shown in Table 1. Therefore, greater amount of BSA can be associated in the QCS/AL nanoparticles, which would lead to relatively high encapsulation efficiency.

Figure 4 shows that as the DS of QCS increased, the encapsulation efficiency increased. Here the QCS samples with different DS have similar molecular weight. The higher DS value means more functional groups on the QCS chains that can complex with BSA, so the encapsulation efficiency increased correspondingly.[26]

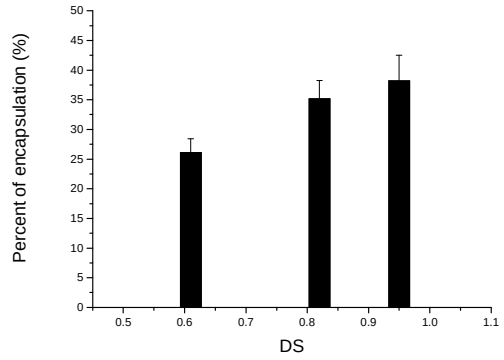

Figure 3. The encapsulation efficiency of BSA in QCS/AL nanoparticles with different DS of QCS(Mw 230 kDa).

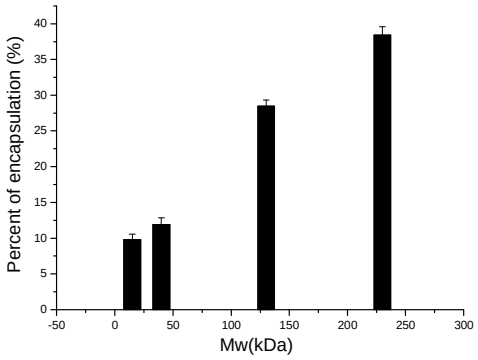

Figure 4. The encapsulation efficiency of BSA BSA in QCS/AL nanoparticles with different Mw of QCS(DS 0.80).

The influence of the initial concentration of BSA on the encapsulation efficiency was shown in Figure 5. Increasing the BSA initial amount increased the encapsulation efficiency from 24 to 36%. The concentration larger than 1 mg/mL didn't lead to further increase of the encapsulation. When the concentration is larger than 1.5 mg/mL, aggregates instead of nanoparticles were formed because of the strong interactions between $–COO^-$ of BSA and $–N^+(CH_3)_3$ of QCS. The encapsulation efficiency was not very high in this study, which can be explained by the method of the nanoparticle preparation. In the nanoparticles formation system, alginate is excessive and the amount is far more than that of QCS. Both BSA and alginate are in their ionic forms and they may

compete in their interaction with QCS. This hinders the encapsulation of BSA into the nanoparticles.

Figure 6 shows that increasing the concentration of QCS can promote BSA encapsulation efficiency, while decrease the load efficiency. As shown previously, increase the concentration of QCS from 1 to 2 mg/mL almost led to a double of the nanoparticle diameter. Therefore, the amount of the BSA entrapment in the nanoparticles increases from 26 to 43% as the concentration of QCS form 0.75 to 1.5% accordingly. On the other hand, the increased QCS adding to the pre-gel effectively enlarged the solid content finally obtained, so the LE decreased with the increased concentration of QCS.

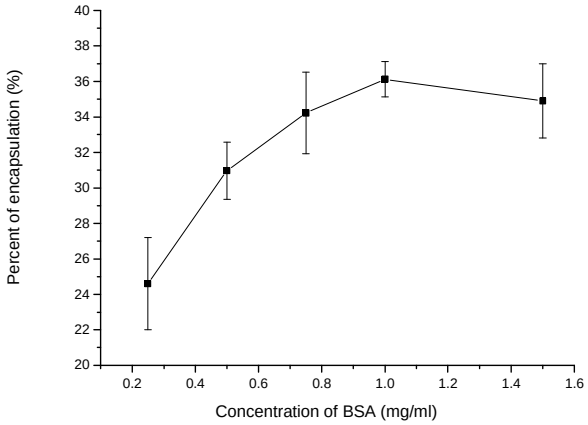

Figure 5. Effect of the concentration of BSA on encapsulation efficiency.

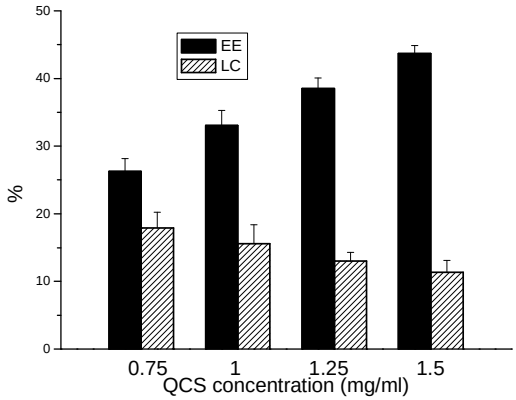

Figure 6. Encapsulation efficiency (EE) and loading capacity (LC) of BSA in nanoparticles under different QCS concentrations.

In vitro release of BSA from nanoparticles

The in vitro release of BSA from the nanoparticles with various Mw and DS of QCS was measured in both simulated gastric fluid (0.1 M HCl) and simulated intestinal fluid (0.1 M PBS, pH 7.4) at 37 °C (Figure 7-8). The release profile was characterized by an initial burst effect followed by a continuous and slow release phase. As shown, the release of BSA from QCS/AL incubated in simulated intestinal fluid is much faster than that for nanoparticles incubated in simulated gastric fluid in the same period. Only 22% BSA was released in 0.1 M HCl while more than 70% of BSA elute out in PBS for the nanoparticles with Mw 230 kDa and DS 0.82 of QCS. The accelerated release of the protein from QCS-Alginate nanoparticles incubated in the high pH media is likely owed to the reduced electrostatic interactions between the polysaccharide-based polyion complexes within the nanoparticles at this pH. The morphology of nanoparticles after 12h's immerse in 0.1 M HCl and 0.1 M PBS are shown in Figure 9. The morphology of nanoparticles doesn't show much change in acid medium(Figure 9a). By contrast, few regular particles were found in PBS due to the disintegration of the alginate matrix in the nanoparticles(Figure 9b). Two processes can explain the release of a drug from a particle: diffusion and erosion. Therefore, in 0.1 M HCl, it seems the process of BSA release was mainly controlled by the diffusion process. In PBS, in addition to the diffusion process, the ion exchanges cause the erosion of the nanoparticles, which greatly increases the BSA release rate.

As shown in Figure 7a, as the Mw of the QCS increased, the release behavior of BSA decreased significantly in 0.1 M HCl. In 3 days, the BSA release percent of nanoparticles with Mw of 230, 130, and 15 kDa were 22, 17 and 9%, respectively. These results indicate that the release behavior of BSA in acid is relative to Mw of QCS solution. It is reported that larger amount of chitosan can bond to alginate microcapsules with decreased molecular mass. [27] Therefore, smaller molecular weight QCS can diffuse more readily within pre-gel and thus form thicker membranes. Since the nanoparticle was comparatively stable in acid, the permeability of the nanoparticles plays an important role in the release of the BSA. The in vitro release profile of BSA loaded QCS-AL nanoparticles with various Mws of QCS in 0.1 M PBS is shown in Figure 7b. The particles showed

a burst release of 60% at 12 h, followed by an additional release of 30% over the next 2 days. All nanoparticles with various Mw showed similar release pattern due to the quick disintegration of nanoparticles in PBS.

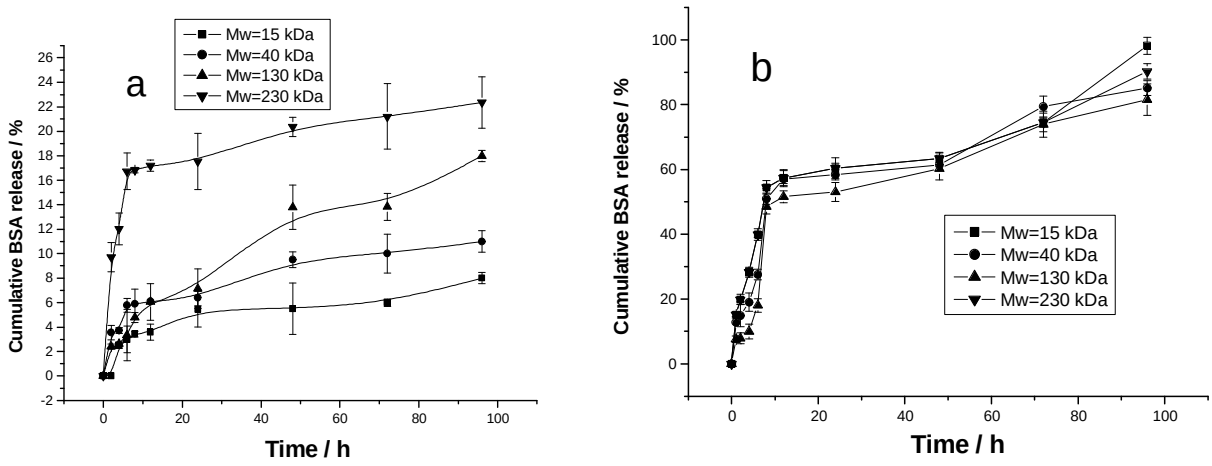

Figure 7. In vitro release of BSA from CS NPs with different Mw of QCS (a) in 0.1 M HCl and (b) in 0.1M PBS (pH = 7.4) (n = 3).

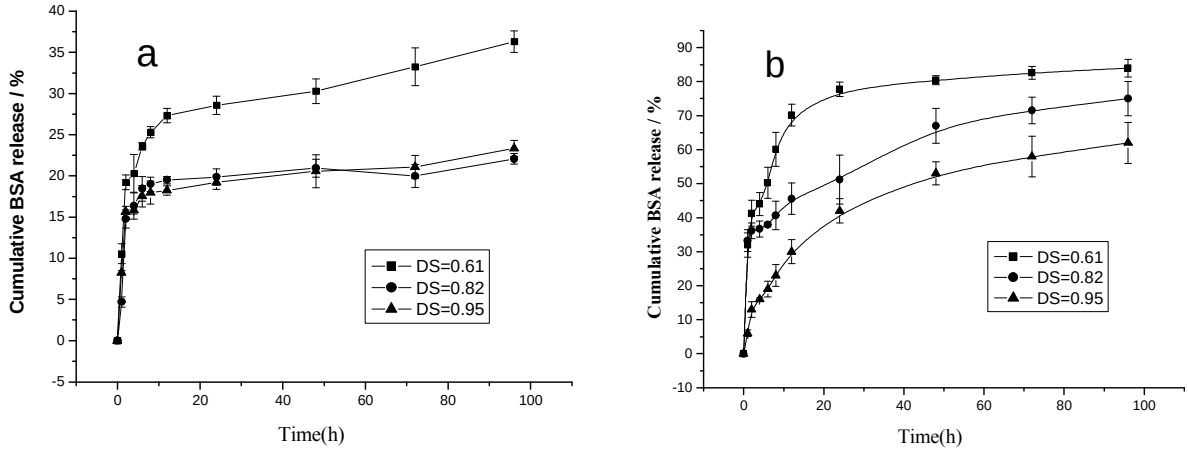

Figure 8. In vitro release of BSA from CS NPs with different DS of QCS (a) in 0.1 M HCl and (b) in 0.1M PBS (pH = 7.4) (n = 3).

The influence of the DS of QCS on the release of BSA from nanoparticles was also investigated (Figure 8). As the DS of QCS increased, a marked decrease of the burst effect and of the release rate was observed, both in 0.1 M HCl (Figure 8a) and in 0.1 M PBS (Figure 8b). This observation that is easily explained by the above mentioned electrostatic interactions between alginate, BSA and QCS, which bind BSA within the nanoparticles. Higher Ds of QCS are expected to provide more compact nanoparticles due to greater number of trimethylammonium groups of QCS interact with alginate. Thus, the release rate pattern of BSA from biodegradable nanoparticles can thus be tailored by choosing the molecular structure parameter of QCS such as Mw and DS.

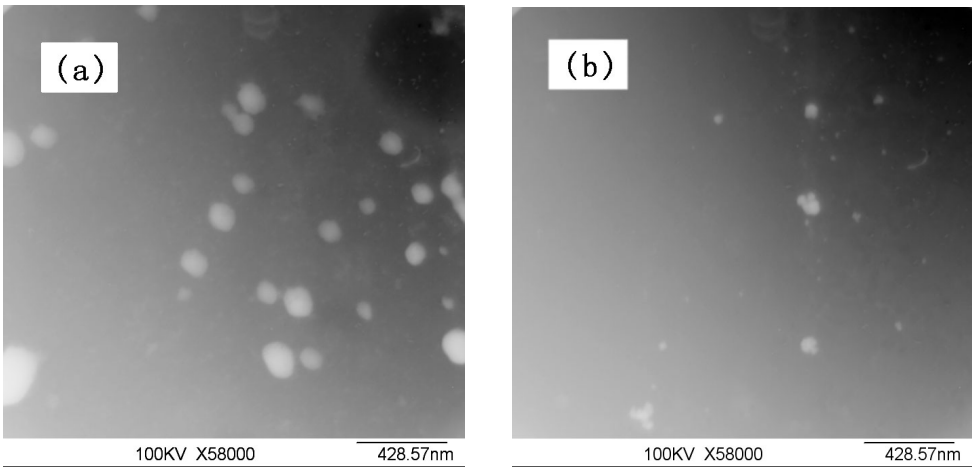

Figure 9. TEM of QCS/AL nanoshperes (a) in 0.1M HCl and (b) in 0.1 M PBS for 12 h.

Conclusions

In the present study, QCS-alginate nanoparticles were prepared as colloidal carriers for the delivery of BSA. Strong electrostatic interactions exist in the nanoparticles. Optimum nanoparticles with a diameter of approximately 200 nm were obtained at QCS:AL:$CaCl_2$ of 6:1:1 (w/w/w) at neutral environment (pH 6.85). Increasing the concentration of BSA from 0.25 to 1.0 mg/mL and QCS from 0.75 to 1.5 mg/mL significantly enhanced the encapsulation efficiency of BSA. The load of BSA was affected by the DS and Mw of QCS as well. The release of BSA from nanoparticles was pH-dependent. Quick release was occurred in PBS compared to that at low pH. The DS and Mw of QCS played an important role in the relese of BSA in vitro. QCS with high Mw accelerate the release of BSA in acid while high DS retian the release of BSA both in 0.1M HCl and in 0.1 M PBS.

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