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Yuhua Chang[a]; Ling Xiao[a]
[a] Department of Environmental Science, College of Resource and Environmental Science, Wuhan University, Wuhan, China

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Preparation and Characterization of a Novel Drug Delivery System: Biodegradable Nanoparticles in Thermosensitive Chitosan/Gelatin Blend Hydrogels

YUHUA CHANG** and LING XIAO*

Department of Environmental Science, College of Resource and Environmental Science, Wuhan University, Wuhan, China

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A novel injectable *in situ* gelling drug delivery system (DDS) consisting of biodegradable N-(2-hydroxyl) propyl-3-trimethyl ammonium chitosan chloride (HTCC) nanoparticles and thermosensitive chitosan/gelatin blend hydrogels was developed for prolonged and sustained controlled drug release. Four different HTCC nanoparticles, prepared based on ionic process of HTCC and oppositely charged molecules such as sodium tripolyphosphate, sodium alginate and carboxymethyl chitosan, were incorporated physically into thermosensitive chitosan/gelatin blend solutions to form the novel DDSs. Resulting DDSs interior morphology was evaluated by scanning electron microscopy. The effect of nanoparticles composition on both the gel process and the gel strength was investigated from which possible hydrogel formation mechanisms were inferred. Finally, bovine serum albumin (BSA), used as a model protein drug, was loaded into four different HTCC nanoparticles to examine and compare the effects of controlled release of these novel DDSs. The results showed that BSA could be sustained and released from these novel DDSs and the release rate was affected by the properties of nanoparticle: the slower BSA release rate was observed from DDS containing nanoparticles with a positive charge than with a negative charge. The described injectable drug delivery systems might have great potential application for local and sustained delivery of protein drugs.

Keywords: Nanoparticles, thermosensitivity, chitosan/gelatin hydrogels, drug delivery systems

1 Introduction

Drug delivery system (DDS) for control drug release was developed encapsulating drugs into a delivery system in order to provide a predetermined drug amount at targeted site over the duration from several hours to several years. In the past few years, an increasing number of *in situ* forming thermosensitive formulations have been reported in the literature for applications in drug delivery system (1, 2). After subcutaneous injection, the cold sol phase containing drugs can form a gel and acts as a depot for sustained release of drugs. Chitosan is an amino-polysaccharide, which derived from the segmental alkaline deacetylation of chitin (3), has many desirable properties, including nontoxicity, biocompatibility, biodegradability, and so on, that attract scientific and industrial interests for numerous applications such as drug delivery, tissue-engineering and gene therapy (4–6). Gelatin is a heterogeneous mixture of hot water-soluble proteins and high average molecular weight, obtained by the thermal denaturation of collagen and possesses excellent properties, so it can be extensively used for pharmaceutical and medical purposes (7, 8). We recently demonstrated that chitosan/gelatin blend solutions neutralized with a small amount of $NaHCO_3$ are thermosensitive. These solutions could retain formulations in solution at neutral pH near 4°C and furthermore allowed gel formation upon heating to body temperature (9). However, protein drug loaded directly into chitosan/gelatin hydrogels was found to release quickly due to the larger pore size of chitosan/gelatin gel network.

Application of nanotechnology for diagnosis, monitoring, disease therapy, and control of biological systems was referred to as "nanomedicine", and it received extensive attentions recently (10). Biodegradable polymeric nanoparticles have attracted considerable attention as potential drug delivery devices in view of their applications in the controlled release of drugs, their ability to target particular organs/tissues, as carriers of DNA in gene therapy, and in their ability to deliver proteins, peptides and genes through a peroral route of administration (11). N-(2-hydroxyl) propyl-3-trimethyl ammonium chitosan chloride

*Address correspondence to Ling Xiao, Department of Environmental Science, College of Resource and Environmental Science, Wuhan University, Wuhan 430079, China. Tel: +86-27-68763162; Fax: +86-27-68763162; E-mail: xiaoling9119@yahoo.cn
**E-mail: changyuhua1122@126.com

(HTCC) can be prepared by a relatively easy chemical reaction of chitosan and glycidyl-trimethyl-ammonium chloride (GTMAC). Quaternized chitosan is potential to be used as an absorption enhancer across intestinal epithelial due to its mucoadhesive and permeability enhancing property (12). Recently, the use of complexation of oppositely charged molecules to prepare HTCC nanoparticles as controlled drug release formulations has attracted much attention, because this process is simple, feasible, and can usually be performed under mild conditions (13). However, nanoparticles may also act at the cellular level. They can be endocytosed/phagocytosed by cells, with a resulting cell internalization of the encapsulated drug (14).

With respect to above considerations, a novel DDS consisting of HTCC nanoparticles and chitosan/gelatin temperature sensitivity hydrogels for prolonged drug release would be preferable. In this paper, protein drugs were loaded into HTCC nanoparticles, then drug-loaded nanoparticles were incorporated physically into chitosan/gelatin hydrogels to obtain a more prolonged drug release DDS. Four different HTCC nanoparticles were prepared based on ionic process of HTCC and oppositely charged molecules such as sodium tripolyphosphate, sodium alginate and carboxymethyl chitosan (CMC), the impacts of the addition of four different HTCC nanoparticles to the characterizations and drug release properties of thermosensitive chitosan/gelatin blend hydrogels were studied.

2 Experimental

2.1 Materials

Chitosan having weight-average molecular weight (M_w) of 1.6×10^5 and degree of deacetylation (DD) of 87% was supplied by Yuhuan Ocean Biochemistry Co. Ltd. (Taizhou, China). Gelatin was obtained from Shanghai Chemical Reagent Co. (Shanghai, China). HTCC with a degree of substitution (DS) of 147% and CMC with a degree of substitution (DS) of 121% were prepared according to the method described by Sun et al. (15). All other reagents used were of analytical grade.

2.2 Preparation of HTCC Nanoparticles

HTCC nanoparticles were prepared by polyelectrolytes complexation method. Briefly, HTCC was dissolved in distilled water, and then oppositely charged molecule aqueous solutions, such as sodium tripolyphosphat, sodium alginate and CMC, were added to HTCC solutions. Three kinds of formations were observed: solution, aggregates and opalescent suspension. The zone of opalescent was further examined as nanoparticles. The composition and preparation conditions of four kinds of nanoparticles based on HTCC are summarized in Table 1. Four kinds of nanoparticles, HTCC-TPP nanoparticles, HTCC nanoparticles modified by alginate, HTCC-CMC nanoparticles with positive charge, and HTCC-CMC nanoparticles with negative charge, were coded as NP1, NP2, NP3 and NP4, respectively. BSA loaded HTCC nanoparticles were prepared at the same way by adding BSA into HTCC solution.

Table 1. The composition and preparation conditions of four kinds of nanoparticles based on HTCC

Sample	*Composition and Preparation Conditions*
NP1	HTCC (2.0 mg/mL); TPP (1.0 mg/mL) $V_{HTCC} : V_{TPP} = 5 : 2$
NP2	HTCC (1.0 mg/mL); $CaCl_2$ (0.5 mg/mL); Na-alginate (1.0 mg/mL) $V_{HTCC} : V_{Na-alginate} : V_{CaCl2} = 1 : 6 : 1$
NP3	HTCC (2.0 mg/mL); CMC (0.5 mg/mL) $V_{HTCC} : V_{CMC} = 1 : 1$
NP4	HTCC (0.5 mg/mL); CMC (2.0 mg/mL) $V_{HTCC} : V_{CMC} = 1 : 1$

2.3 Preparation of Chitosan/Gelatin Blend Hydrogels Containing Nanoparticles

Chitosan solutions were obtained by dissolving 200 mg of CS in 0.1 M HCl (10 mL) and chilled in an ice bath for 15 min. Gelatin was added to deionized water and heated at 50°C for 2 h to make solutions containing 1% w/v gelatin. The gelatin solution (1 mL) was dropped into the chitosan solution in an ice bath under magnetic stirring and was stirred for 15 min to gain homogeneous mixture. Then, the cooled 1mL $NaHCO_3$ (1.0 M) was dropped into the stirring chitosan/gelatin solutions in an ice bath to neutralize solution to neutral pH. The nanoparticles were added into chitosan/gelatin solutions, and the obtained solutions were stirred for another 30 min to gain homogeneous mixture. The hydrogel was formed by heating the solutions in a water bath at 37°C for a few minutes. The hydrogel without nanoparticles was coded as G0, hydrogel containing NP1, NP2, NP3, NP4, was coded as GNP1, GNP2, GNP3, GNP4, respectively.

2.4 Characterization

FTIR spectra of CS, HTCC, CMC, HTCC nanoparticles were recorded in KBr pellets in the range of 4000-400cm^{-1}on a Nicolet FTIR 5700 spectrophotometer (Madison, WI) at room temperature. The particle size and morphological measurements of the HTCC nanoparticles were performed by TEM-100 CXII (Electronic Company, Japan). Zeta potential measurements of nanoparticles were performed by Zetasizer Nano ZS (Malvern, England).

The gelation time of solution was determined by test tube inverting method (16). The obtained formulation in solution state (12 mL) was added into a tube (25 mL) with a glass cap and kept in a water bath at 37°C. At

predetermined interval, the tube was take out and inverted to observe the state of the sample. The gelation point was determined by flow or no-flow criterion over 30 s with the test tube inverted.

Gel strength was measured by the following method. The plexiglass rod with cross sectional area of 1 cm^2 was fixed in a vertical support, and contacted with the surface of gel in the balance left plate on the tray-beaker. The weight was slowly added in the right plate up to the gel surface rupture, at this time, the weight in the right plate responses to gel strength.

The morphological measurements of hydrogels were observed after lyophilization. The samples were sputter-coated with gold and imaged on a Hitachi S-570 Scanning Electron Microscope (Tokyo, Japan) using an accelerating voltage of 20 kV.

The degree of swelling (Ds) of dry gels was determined by immersing the dry gels in pH 7.4 phosphate buffered saline (PBS) solutions at room temperature. At predetermined time intervals, they were removed from the solutions, gently wiped with filter paper to remove the surface solution, weighed and returned to the same container until equilibrium was achieved. The Ds was determined according to the following equation:

$$D_S = (W_S - W_0)/W_0,$$

Where W_0 is the weight of dry gel, W_s is the weight of gel at different swelling time.

2.5 *In vitro* Release of BSA

BSA loaded HTCC nanoparticles were added in the stirring chitosan/gelatin blend solutions in an ice bath. Then the solutions were incubated at 37°C for 10 min to form hydrogels. BSA-loaded hydrogels were immersed in 5 mL PBS buffer with pH 7.4 at 37°C in a thermostated shaker. At predetermined intervals, 1 mL of the PBS buffer was taken out and the release of BSA was estimated. With each sample, the solution was changed with fresh medium, maintaining the total volume constant. The hydrogel containing nanoparticles without BSA was the blank and it was measured at the same way as comparable sample. BSA released from the hydrogel could be measured by UV-9100 spectrophotometer (Beijing Rayleigh Analytical Instrument CO., China) at 595 nm with Coomassie Brilliant Blue G-250. The percentage of cumulative amount of released BSA was determined from standard curves.

3 Results and Discussion

3.1 Physicochemical Characterization of HTCC Nanoparticles

Figure 1 shows the FTIR spectra of CS, HTCC, CMC, HTCC nanoparticles. Chitosan can be seen in a typical three peaks: 3435 cm^{-1}, 1637 cm^{-1} and 1088 cm^{-1}, respectively, attributable to the ν (OH), δ (NH2), ν (COC). In the spectrum of HTCC, the characteristic peak (1637 cm^{-1}) representing NH_2 deformation is weakened and a new peak positioned at 1483 cm^{-1} is appeared, which corresponds to an asymmetric angular bending of methyl groups of quaternary hydrogen. The characteristic peaks of primary alcohol and secondary alcohol between 1102 and 1082 cm^{-1} are not change in HTCC comparing with chitosan that proves the introduction of quaternary amino groups at NH_2 sites on chitosan chains. Compared with HTCC, the FTIR spectrum of HTCC-TPP nanoparticles has a more broad peak at around 3424 cm^{-1} corresponding to the stretching vibration of -NH_2 group and -OH group, it indicates that hydrogen-bonding forces increasing in the nanoparticles. Besides, the N-H-bending vibration in 1640 cm^{-1} transfers to the 1648 cm^{-1}, which indicates the electrostatic attractions via the phosphate groups of TPP and ammonium of HTCC. The characteristic peak of free hydroxyl of HTCC transfers from the original 1260 cm^{-1} to 1225 cm^{-1}, which indicates the free hydroxyl forms the molecular hydrogen bonding in HTCC-TPP nanoparticles (NP1). In the infrared spectrum of HTCC nanoparticles modified by alginate (NP2), the asymmetrical stretching peak of carboxyl group transfers to 1611 cm^{-1}, symmetrical stretching peak shifts to 1420 cm^{-1}, which indicates carboxyl groups of the alginate are ionized to COO^-, and can form electrostatic attractions with the quaternary groups with positive charge. At the same time, the board peak at 1483 cm^{-1}becomes into the shoulder peaks and has a significant reduction in strength, which shows the strong interaction between HTCC and alginate in the nanoparticles. Two strong peaks at 1627 and 1413 cm^{-1} (in CMC spectrum) are observed due to the asymmetrical and symmetrical stretching of COO– group. In the spectrum of CMC, the C–O stretching band at 1030 cm^{-1} corresponding to the primary hydroxyl group disappears, verifying a high carboxymethylation of OH-6. The characteristic peak

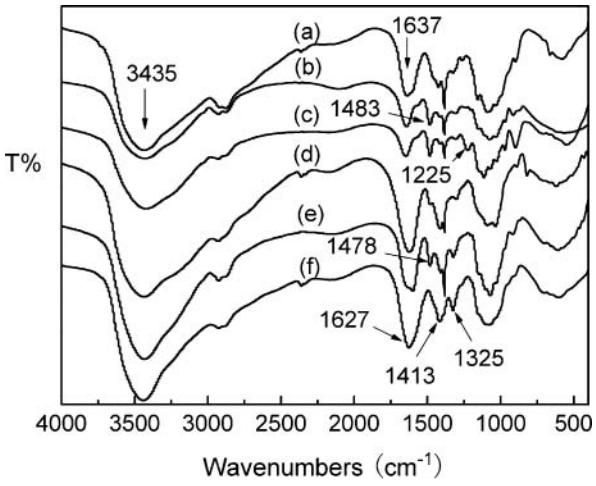

Fig. 1. FTIR spectra of (a) chitosan; (b) HTCC; (c) NP1; (d) NP2; (e) NP3; (f) CMC.

Table 2. Sizes and charges of HTCC nanoparticles

	NP1	*NP2*	*NP3*	*NP4*
Mean Size (nm)	248	200	300	325
Zeta Potential (mV)	12.7	11.2	13.9	–11.3

of second hydroxyl group at 1080 cm^{-1} is not changed. Compared with HTCC and CMC, the FTIR spectra of HTCC-CMC nanoparticles (NP3) has a more broad peak at around 3431cm^{-1} corresponding to the stretching vibration of -NH_2 group and -OH group, it indicates that hydrogen-bonding forces increasing in the nanoparticles. Besides, the N-H-bending vibration in 1640 cm^{-1} transfers to the 1600 cm^{-1}, which indicates the electrostatic attractions via $–N^+(CH_3)_3$of HTCC and $–COO^-$ of CMC.

Figure 2 shows the morphological characteristic of four different HTCC nanoparticles. Four different HTCC nanoparticles all have spherical shape and monodisperse. Table 2 shows the sizes and zeta potentials of four different HTCC nanoparticles. NP1 is about 248 nm in size and the zeta potential is 12.7 mV, which shows the nanoparticles surface is positively charged. NP2 is 200 nm in size and more spherical and smoother, which may be due to its compact structure. Alginate plays different roles in formation of nanoparticles. Alginate is anionic polymer, its COO^-groups can interact strongly with $–N^+(CH_3)_3$ groups of HTCC, and the similarity of the polysaccharide structure of two biopolymer offers a great interaction, which results in a strong interchain reaction and more compact formation. The zeta potential of the NP2 is 11.2 mV, which shows the nanoparticles are positively charged. NP3 is about 300 nm in size and the zeta potential is 13.9 mV. HTCC-CMC nanoparticles with negative charge (NP4) are about 325 nm in size and the zeta potential is –11.3 mV.

3.2 Preparation of Chitosan/Gelatin Blend Hydrogels Containing Nanoparticles

Chitosan is not soluble in water, but chitosan solutions can be obtained in acidic aqueous media. Further basification, to pH > 6.2, systematically leads to the formation of a hydrated gel-like precipitate. In our previous study, the chitosan/gelatin solutions could retain liquid state at neutral pH in an ice bath, and form gel upon heating to 37°C. The addition of gelatin is to prevent precipitation of chitosan solution and controls the hydrogel formation when an increase in temperature is imposed. When HTCC nanoparticles were added into the chitosan/gelatin solutions, the hydrogels also could be formed at 37°C. The gelation mechanism of the chitosan/gelatin hydrogel containing nanoparticles is illustrated in Figure 3. In the systems, three types of interactions may be involved during the gelation process: (1) hydrogen bonding between the polymer chains; (2) hydrophobic interactions between

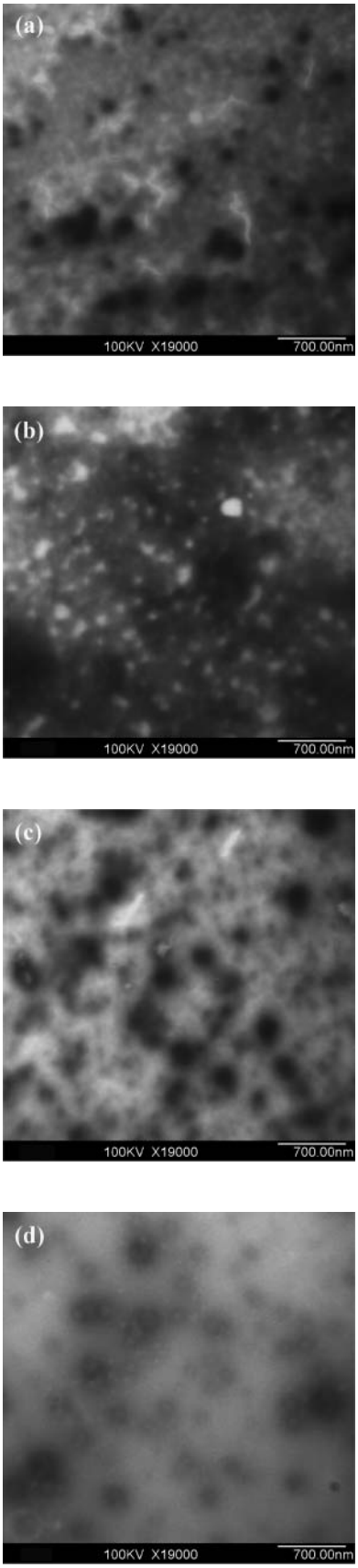

Fig. 2. TEM of (a) NP1; (b) NP2; (c) NP3; (d) NP4.

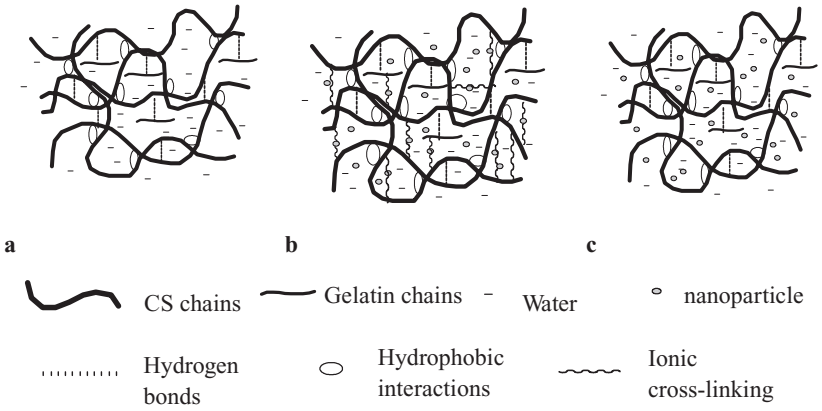

Fig. 3. The schematic illustration of the formation mechanism of: (a) chitosan/gelatin blend hydrogel; (b) hydrogel containing nanoparticles with positive charges; (c) hydrogel containing nanoparticles with negative charges.

polymer chains; and (3) the ionic crosslinking between polymer chains and nanoparticles. The gel formation of the system is the competition result of these three types of interactions in hydrogel solutions.

3.3 Influence of Nanoparticles on Gelation Time and Gel Strength

Table 3 shows the influence of four different HTCC nanoparticles on gelation time and gel strength. In this study, the concentration of nanoparticles (1 mg/mL) in solution was confirmed to go on the following experimentation. Gelation time was shortened and gel strength was increased by adding of nanoparticles with positive charges (NP1, NP2, NP3) into chitosan/gelatin solutions. However, the gelation time was increased and gel strength was decreased by adding of nanoparticles with negative charges (NP4). The incorporation of nanoparticles with positive charges shortens gelation time and increases the mechanical properties. This is attributed to the existence of nanoparticles with positive charges, which act like reinforced nodes, short the distance of interaction point in polymer chains and accelerate chain aggregation, enhance the interactions between the polymer chains and increase the strength of resulting network. The adding of nanoparticles with negative charges increases the inter-chain electrostatic repulsion and reduces the interactions between the polymer chains, results the strength of network lower.

Table 3. Influence of HTCC nanoparticles on gelation time and strength of chitosan/gelatin hydrogels

Gel samples	*Samples containing nanoparticles*	*Gelation time (min)*	*Gel strength ($g \cdot cm^{-2}$)*
G0	Gel without nanoparticles	7	22.5
GNP1	Gel containing NP1	5	33.6
GNP2	Gel containing NP2	5	34.8
GNP3	Gel containing NP3	6	26.4
GNP4	Gel containing NP4	8	19.3

3.4 Scanning Electron Microscopy

Marco-porous gels can be examined using scanning electron microscopy (SEM) after freeze drying when the material has an adequate modulus to avoid the structural collapse during dehydration. Figure 4 shows SEM of blank chitosan/gelatin gel and chitosan/gelatin gels containing HTCC nanoparticles, the SEM micrographs are further magnified to clearly show nanoparticles in gels in Figure 4(b,c,d,e). These images indicate that chitosan/gelatin gel and chitosan/gelatin gels containing nanoparticles have similar interconnection pore structure forming a three-dimensional network structure, no obvious difference of morphology is presented. HTCC nanoparticles, regardless of the charges and composition, did not have significant influence on the morphology of the chitosan/gelatin gel network. This may be explained from the gelation mechanism of chitosan/gelatin solution. It has been demonstrated that the gelation mechanism of chitosan/gelatin solution was nucleation and growth. During nucleation, the polymeric phase is composed of many small regions, which during the growth phase will expand and connect (9). Chitosan/gelatin hydrogel was found to have a beaded, open structure, forming a network by linking polymeric aggregates in agglomerates and chains. The polymer-rich phase increased with increasing of polymer content. In this study, the amount of HTCC nanoparticles added into the system was little compared with the amount of polymer, so HTCC nanoparticles did not have significant influence on the morphology of the chitosan/gelatin gel network although interactions exist between the nanoparticles and the polymer chains.

3.5 Swelling Behavior

The swelling behavior of chitosan/gelatin dry gel containing nanoparticles was studied as a function of the

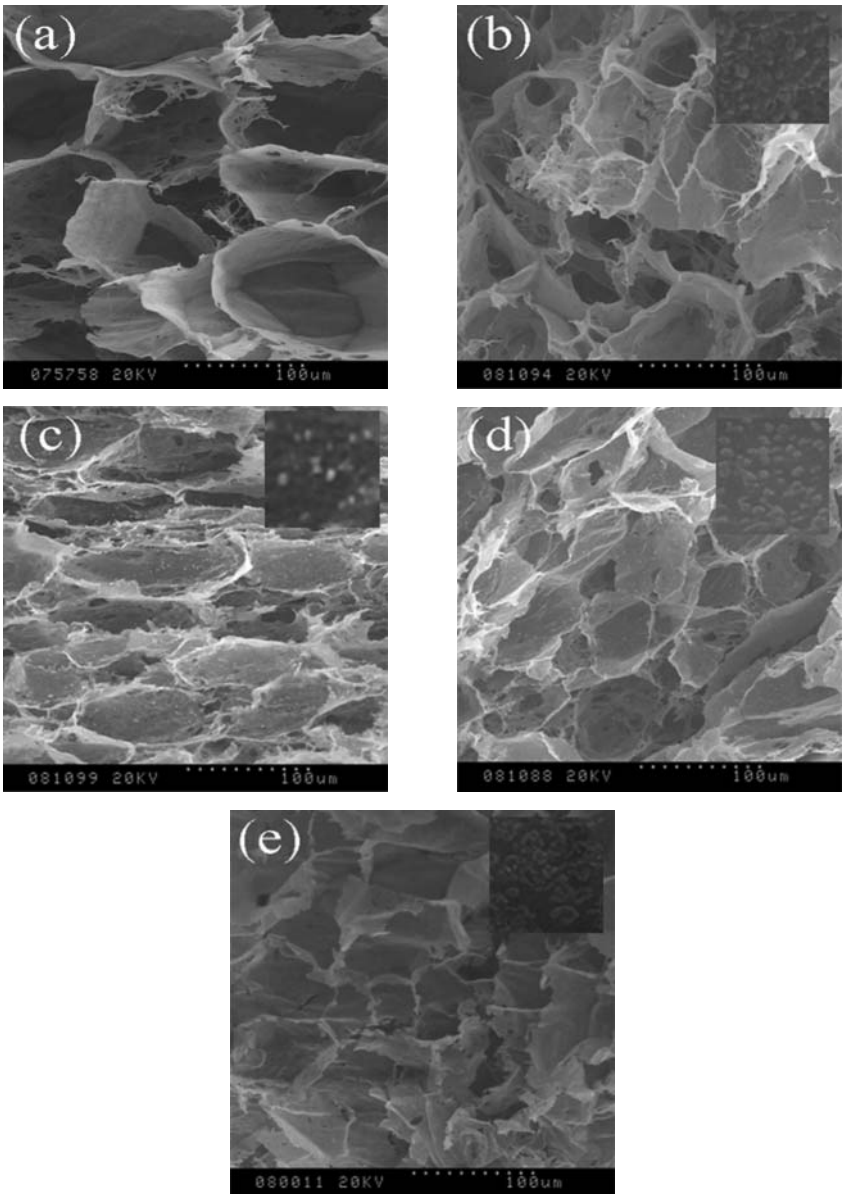

Fig. 4. SEM of chitosan/gelatin blend hydrogels: (a) G0; (b) GNP1; (c) GNP2; (d) GNP3; (e) GNP4.

hydrogel properties. The results of swelling experiments of chitosan/gelatin dry gel containing nanoparticles under pH 7.4 PBS buffer were shown in Figure 5. The chitosan/gelatin dry gel containing nanoparticles with positive charges swells more rapidly than the chitosan/gelatin dry gel without nanoparticles, but the swelling ratio of chitosan/gelatin dry gel containing nanoparticles with positive charges is smaller than that of chitosan/gelatin dry gel. The swelling properties of dry gel can be explained as follows. The chitosan/gelatin dry gel swells because low gelatin concentration leads to weak hydrogen bonding and consequent facilitation of the entrance of solvent into the material. The hydrogels containing nanoparticles with positive charges swell quickly because the hydrophilic property of HTCC nanoparticles. The swelled network allows more solutions to penetrate into the gel. However, the existence of nanoparticles with positive charges, which act like crosslinking nodes, enhances the interactions between the polymer chains and results the gel swelling degree decrease. The gel containing nanoparticles with negative charges behaves quite differently, it swells at first quickly and then shrinks, the swelling ratio is at first larger and then becomes lower than that of chitosan/gelatin dry gel with and without positive charges. The hydrogel swells quickly because the hydrophilic property of HTCC nanoparticles, the swelled network allows more solutions to penetrate into the gel. The adding of nanoparticles with negative charges increases the inter-chain electrostatic repulsion and reduces the interactions between the polymer chains, so the swelling ratio is larger than that of

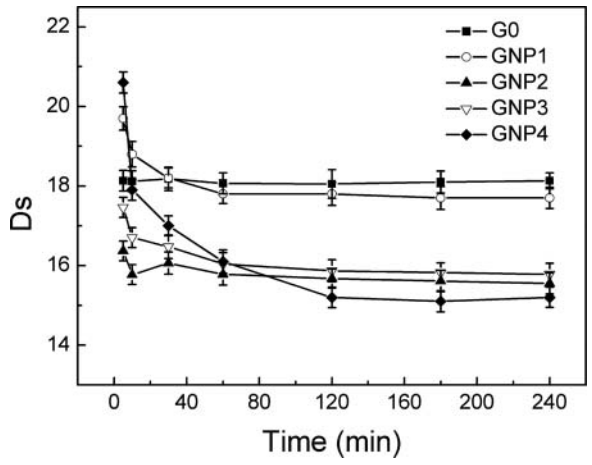

Fig. 5. Swelling ratio of gels with different nanoparticles in pH 7.4 PBS buffer (n = 3).

chitosan/gelatin dry gel with and without positive charges. The swelling ratio decreases later might due to that a part of phosphate ions in solution diffuses slowly into the gel network, masks the negative charges of nanoparticles in gel and strengths the coiled chain to a more narrow size.

3.6 *In vitro* Release of BSA

Protein drugs are increasingly becoming a very important class of therapeutic agents with the rapid advances in the field of biotechnology currently. These drugs are mostly delivered by parenteral administration. However, repeated injections are required due to extremely short acting of this kind of drugs. To minimize the health hazard by constant injection, sustained delivery is the ideal alternate route of administration (17). Figure 6 shows the BSA cumulative release profiles from chitosan/gelatin gels with four different BSA loading HTCC nanoparticles in pH 7.4 buffer at 37°. The release rate of BSA from gels is affected by the properties of nanoparticle. The adding of four different HTCC nanoparticles all slows the release rate of BSA. The mechanism of drug release may be the diffusion of BSA through the three-dimensional network, the smaller pore size of the network results in a slower release rate of the drug loaded. Besides, the interaction between BSA and positively charged nanoparticles should play an important role to decrease the release rate of BSA from the gel. *In vitro* release of BSA from these polymer hydrogels were sustained over 10 days without a distinct initial burst. The slower BSA release rate was observed from hydrogels containing nanoparticles with positive charge than with negative charge. BSA release is more rapid from the gel containing HTCC nanoparticles with negative charges compared with the gel containing HTCC nanoparticles with positive charges; this is due to electrostatic repulsion of negative charges between HTCC nanoparticles and BSA accelerate BSA release. These results demonstrate that thermosensitive chitosan/gelatin gels with different nanoparticles, particularly nanoparticles with positive charges, are potential to use as a vehicle for the delivery of proteins.

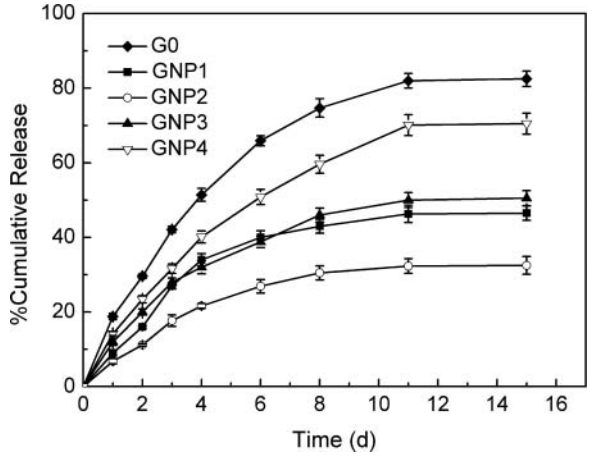

Fig. 6. Accumulative release of BSA from gels with different nanoparticles in pH 7.4 PBS buffer (n = 3).

4 Conclusions

In this paper, we have successfully developed a novel injectable drug delivery system consisting of biodegradable HTCC nanoparticles and thermosensitive chitosan/gelatin blend hydrogels for sustained controlled drug release. The addition of four different HTCC nanoparticles into chitosan/gelatin gel system all slowed the BSA release rate. The results also showed that the release rate of BSA from gels was affected by the properties of nanoparticle, the slower BSA release rate was observed from DDS containing nanoparticles with a positive charge than with a negative charge. These demonstrated thermosensitive chitosan/gelatin gels containing HTCC nanoparticles have a great application as a vehicle for the local controlled delivery of proteins.

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