Archives of Razi Institute, Vol. 67, No. 2, December (2012) 145-153

Full Article

Preparing and Characterizing Chitosan Nanoparticles Containing *Hemiscorpius lepturus* Scorpion Venom as an Antigen Delivery System

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Received 12 Nov 2011; accepted 13 Apr 2012

ABSTRACT

In recent years, chitosan nanoparticles have been studied widely for protein delivery. In this study, *Hemiscorpius lepturus* (*HL*) venom was encapsulated in chitosan nanoparticles. The aim of the present work was to carry out a systematic study for preparing biocompatible and biodegradable nanoparticles for loading *HL* scorpion venom and to evaluate their potential as an antigen delivery system. In this study, *HL* venom loaded chitosan nanoparticles fabricated by ionic gelation of chitosan and tripolyphosphate and the factors which may be influenced in the preparation of nanoparticles were analyzed. Also, their physicochemical properties and in vitro release behavior were studied. The optimum encapsulation efficiency and capacity were observed when the chitosan concentration and *HL* venom were 2mg/ml and 500µg/ml, respectively. The *HL* venom loaded nanoparticles were in the size range of 130-160nm (polydispersity index values of 0.423) and exhibited the positive zeta potential. Transmission electron microscope imaging showed spherical and smooth surface of nanoparticles. The profiles of the release exhibited a burst releases about 50% in the first 4 hr and then slowed down at a constant rate. The obtained results suggested that the chitosan nanoparticles prepared in this work had the potential for antigen delivery.

Keywords: Chitosan, Nanoparticle, Venom, Ionic gelation

INTRODUCTION

A new and advanced method is necessary for improving the hyper-immunization process of animals in order to produce antivenoms. In this regard, it is important to reduce the number of repeated venom administrations required for long-term immunization and increase the production yield. Enormous efforts have been made in order to use biodegradable polymers as controlled-release systems for the development of vaccines vehicle (Aguado & Lambert 1992, Langer & Folkman 1976). In the previous studies, microcapsules or microspheres were used for the controlled delivery

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systems (Kohn *et al* 1986, Eldridge *et al* 1991). The mechanism of antigen release from biodegradable microspheres is a combination of surface and bulk erosion of the polymer, diffusion of the antigen through the polymer matrix and penetration of the antigen through the pores. The ideal controlled-release system should deliver venom in such a way that a long-lasting boosting effect is achieved by a single administration and provide effective antibody responses against the delivered agent. Nanoparticles are solid colloidal particles with diameters ranging from 1 to 1000 nm. They consist of macromolecular materials which can be used therapeutically as an adjuvant in vaccines or drug carriers in which the active ingredient is dissolved, entrapped, encapsulated, adsorbed or chemically attached. Both the synthetic and natural polymers can be used for preparing nanoparticles. Natural polysaccharide polymers such as chitosan (CS) have been extensively studies in the recent years as a primary material in synthesizing carriers for therapeutic protein molecules and as non-viral gene carrying vectors (Douglas et al 2006, George & Abraham 2006, Dang & Leong 2006).

Chitosan, a (1-4)-linked 2-amino-2-deoxy-b-D-glucopyranose, is derived from chitin, (1-4)-linked2-acetamido-2-deoxy-b-D-glucopyranose. CS can be degraded into *N*-acetyl glucosamine by general lysozymes in the body, which is subsequently excreted as carbon dioxide via the glycoprotein synthetic pathway. Chitosan is widely used in pharmaceutical and medical purposes due to its advantages and properties such as biodegradability, biocompatibility, low toxicity, haemostatic, bacteriostatic, fungistatic, anticancerogen and anticholesteremic properties (Hejazi & Amiji 2003). CS also acts as a penetration enhancer by opening the tight epithelial junctions; therefore, it is particularly exploited in protein and vaccine delivery (Van der Lubben *et al* 2001a, Van der Lubben *et al* 2001b). CS nanoparticles have been widely investigated for the delivery of polypeptides such as insulin (Avadi *et al* 2010), tetanus toxoid (Vila *et al* 2004), diphtheria toxoid (Rezaei Mokarram & Alonso 2006, Van der Lubben *et al* 2003), snake venom (Mohammadpour dounighi *et al* 2010) and proteins (Xu & Du 2003, Calvo *et al* 1997, Calvo *et al* 1997, Janes & Alonso 2003).

Chitosan nanoparticles have been made by chemical cross-linking with glutaraldehyde, glyoxal and ethylene glycol diglycidyl ether (Akbuga & Durmaz 1994, Berger *et al* 2004). Although these are very good cross-linkers, they are not preferred owing to their physiological toxicity. CS is polycationic in acidic media (pK_a 6.5) and can interact with negatively charged species such as sodium Tripolyphosphate (TPP) and sodium sulfate. Because of this characteristic, TPP can be used for preparing cross - linked CS nanoparticles. The interaction of CS with TPP leads to the formation of biocompatible cross-linked CS nanoparticles, which can be efficiently employed in protein and vaccine delivery. In addition, the deacetylated CS backbone of glucosamine units has a high density of amine groups, leading to electrostatic interactions with proteins and genes which carry overall negative charges at natural pH conditions (MacLaughlin *et al* 1998, Mao *et al* 2004). On the other hand, some studies have focused on synthesizing CS thiomers that have higher water solubility at the pH above 6 and better mucoadhesive properties than CS. These advantages make thiomer derivatives of CS highly interesting for safe drug delivery systems (Calvo *et al* 1997a, Calvo *et al* 1997b, Aghaei Moghaddam *et al* 2009).

The aim of the present study was to prepare biocompatible and biodegradable nanoparticles for loading *Hemiscorpius lepturus* scorpion venom and to evaluate their potential as antigen delivery system. A series of CS nanoparticles were prepared and factors which may influence the preparation of nanoparticles were analyzed. Furthermore, physicochemical properties and *invitro* release behavior of nanoparticles were studied.

MATERIALS AND METHODS

CS from a shrimp shell (low molecular weight

[LMW], Deacetyl Degree [DD] 85%) was purchased from Primex (Siglufjordur, Iceland). Sodium tripolyphosphate (TPP) and Coomassie Blue G250 were purchased from Sigma-Aldrich (St.Louis, Missouri). Acetic, Phosphoric acid and Ethanol were purchased from Merck (Darmstadt, Germany). *HL* venom was provided as a freeze-dried powder by Razi Vaccine and Serum Research Institute (Karaj, Iran). The other materials used in this study were of analytical grade.

Preparing *HL* Venom Chitosan Nanoparticles. Chitosan nanoparticles (CS NPs) were prepared mainly according to the procedure reported by Calvo *et al* (Atyabi *et al* 2008, 2009) with little modification based on the ionotropic gelation (Kawashima *et al* 1985a, Kawashima *et al* 1985b, Nishioka *et al* 1990, Werle *et al* 2009) of CS with TPP anions. CS with different concentrations were dissolved (1.5, 2.0, 3.0 mg/ml) in an acetic aqueous solution. The concentration of acetic acid in the aqueous solution was 1.5 time higher than that of CS in all cases. The TPP was dissolved in distilled water at the concentration of 1.0 mg/ml. 2 ml of TPP solution was added to 5ml of the CS solution under magnetic stirring (1000rpm, 60 minute) at room temperature and opalescent suspension was formed spontaneously; finally, nanoparticles were separated by centrifugation at 12000 ×g for 30min at 5±3 °C. After centrifugation, the sediment of nanoparticles was freeze dried (-60 °C, 0.08 mbar) and stored at 4-8 °C until being analyzed. The *HL* venom loaded nanoparticles were formed upon the incorporation of TPP solution (containing various concentrations of *HL* venom) to the CS solution.In this work, the effects of some factors such as venom concentrations (21, 43, 64, 500 and 750µg/ml) and Chitosan concentrations (1.5, 2 and 3 mg/ml) on the characteristics of nanoparticles were also investigated. For evaluating one factor, only its own parameter was changed and other factors remained constant.

Characterizing the Nanoparticles. The morphology of the particles were examined by transmission electron microscope (TEM) (Philips 400, kV 80; Eindhoven, Netherland). The samples were immobilized on copper grids, colored with Phospho-Tungestic acid (PTA), dried at room temperature and then examined by TEM (Van der Lubben *et al* 2003). The particle size and zeta potential were evaluated by a dynamic light scattering (DLS) technique as performed by Zeta sizer (SEM-633 SEMTech). The particle size and particle size distribution were reported as an intensity and a polydispersity index (PdI), respectively (Avadi *et al* 2010).

Structure of Nanoparticles. The IR spectra of the samples were taken with KBr pellets and recorded by Fourier-transformed infrared spectrophotometer instrument (FTIR- 410 Jasco Colchester, United Kingdom) with attenuated total reflection (ATR).

Venom Encapsulation Efficiency and Loading Capacity. The protein concentration was estimated by Bradford method at 595nm. The amount of venom encapsulated in the nanoparticles was measured by calculating the difference between the total amounts of the venom added in the nanoparticle preparation solution and the amount of nonentrapped venom remaining in the clear supernatant after the centrifugation of samples at 12000 ×g for 30min at 5±3 °C. The amount of free venom in the supernatant was estimated by the above mentioned method. The *HL* venom encapsulation efficiency (AE) and loading capacity (LC) were calculated according to the following equations (Van der Lubben *et al* 2003):

$$\%AE = \frac{(\text{total amount of } HL \text{ venom}) - (\text{free amount of } HL \text{ venom})}{\text{total amount of } HL \text{ venom}} \times 100$$

$$\%LC = \frac{(\text{total amount of } HL \text{ venom}) - (\text{free amount of } HL \text{ venom})}{\text{nanoparticles' weight}} \times 100$$

In Vitro Release Studies. *HL* venom release profile from CS NPs was determined as follows. A certain amount of nanoparticles was suspended in 3ml of release media (PBS 0.2 mol/lit, pH 7.4) and incubated in a shaking water bath at 37±1°C, 50rpm. The 100µl samples were taken from the tube and centrifuged at predetermined time intervals of 2, 4, 6, 8, 16, 24, 48

and 56hr after incubation .The samples in the tube were replenished with 100µl fresh PBS solution at 37±1°C. The protein concentration in the supernatant was measured by Bradford method. Triplicate samples were analyzed and the total released protein mass M_i at time i was calculated as follows: $M_i = C_iV + \Sigma C_{i-1}V_s$ where C_i is the concentration of protein in the release solution at time i, V is the total volume of release media and V_s is the sample volume.

RESULTS

Physicochemical Characterization of Nanoparticles. The *HL* venom loaded CS NPs instantaneously formed, when solution of polyanionic TPP and *HL* venom is added to the CS solution. TEM of the nanoparticles and their morphology are shown in Figure 1. The shape of the particles was approximate to spheres with almost homogeneous structure. The particles size of nanoparticles was analyzed by TEM and zetasizer (Figures 1 and 2). As it is shown in the above mentioned figures, the size range of *HL* venom loaded CS-TPP nanoparticles was approximately 137nm under TEM and 157.7 nm (with the relatively narrow particle size distribution and low PdI values 0.423) by zetasizer. Moreover, the zeta potential of the chitosan nanoparticles and *HL* venom loaded chitosan nanoparticles were measured to be 39.2 and 35.4 mv, respectively (Figures 3A and 3_B). FTIR spectra of chitosan nanoparticles and chitosan are shown in Figures 4A and 4_B. In the CS spectra, the strong and broad bond in the 3200-3450cm^{-1} range corresponded to the amine and hydroxyl groups; the peak at near 2858 cm^{-1} was caused by -OH stretching. The absorption band of the carbonyl (C=O) stretching of the secondary amide (amide I band) was observed near 1658 cm^{-1} as well. The intense peak at 1374 cm^{-1} belonging to the N─H stretching of the amide gave the peak in the fingerprint region of the spectra, in which the symmetric stretch of C─O─C was found around the wave number of 1087 cm^{-1}.

Encapsulation of *HL* Venom within Nanoparticles. The results obtained in the present study indicated that, by increasing the venom concentration (from 64 to 700µg/ml), both %AE and %LC increased (Table 1). Table 1 shows by increasing the CS concentration from 1.5mg/ml to 3mg/ml, the %AE and %LC increased. The optimum encapsulation efficiency and loading capacity were observed for nanoparticles prepared in conditions 2mg/ml of CS and 500µg/ml of venom concentration.

In Vitro Release. In vitro release of the venom loaded CS NPs was evaluated by using the NPs prepared in optimum conditions (2mg/ml CS and 500µg/ml of venom concentration) with the loading capacity of 88.84%. The release behavior is shown in Figure 5. The profile of release exhibited a burst releases about 50% in the first 4 hr; then it got slower at a constant rate.

DISCUSSION

The *HL* venom engagement with a long chain of CS molecules was not uniform and less spread. CS molecules were likely to adopt a different conformation in the solution due to the electrostatic interactions existing between amine groups along the molecular chain.

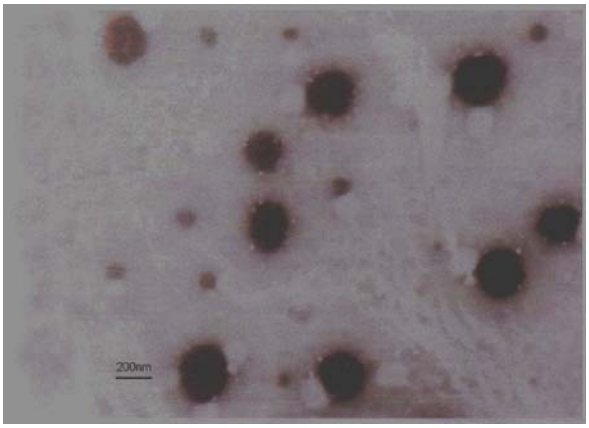


Figure 1. TEM image of the Chitosan nanoparticles containing *Hemiscorpius lepturus* (*HL*) venom (LMW CS 2mg/ml, TPP 1mg/ml and *HL* Venom 500µg/ml).

The carboxylic groups on the surface of the large protein molecules can be create hydrogen bonds with amine groups at specific parts of the CS chain while maintaining a compact 3-D structure at pH 5.4. Therefore, the inner hydrophobic core of protein molecules remained unchanged (Mao *et al* 2001). The surface morphology of nanoparticles (shown in Figure 1) was confirmed with the above mentioned report.

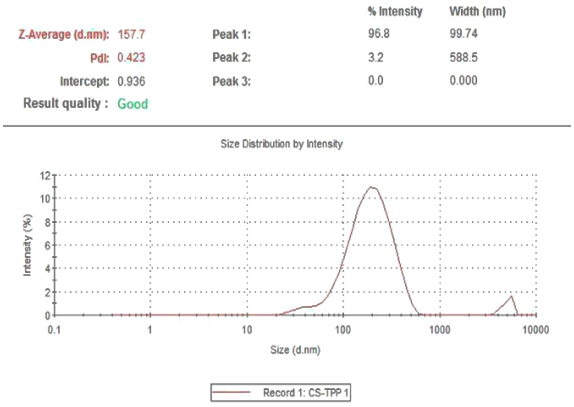


Figure 2. Size analysis of *Hemiscorpius lepturus* venom loaded chitosan nanoparticles by DLS.

As shown in Figures 1 and 2, the diameter size of particles measured by zetasizer (157.7nm) correlated with the results obtained with TEM (137nm). The PdI of nanoparticles was smaller than 0.5 (Figure 2) which showed relatively homogeneous dispersion. It can be also concluded that the loading process of *HL* venom could not considerably change the size and shape of the *HL* venom loaded CS NPs (Figures 1 and 2) than venom free nanoparticles. The zeta potential of the particles only slightly decreased from 39.2 to 35.4 mv by loading the *HL* venom (Figures $3_{A,}$ 3_{B}). The results show that the protein molecules entrappment does not sufficiently suppress the positive surface charge of CS molecules, when the z-potential profile of CS NPs is compared with the z-potential of CS NPs containing *HL* venom. It still seems that a high proportion of free amine groups on the CS chain remained unoccupied and it can be proposed that the zeta potential of CS and venom loaded nanoparticles can influence their stability in suspension through electrostatic repulsion between the particles. As FTIR spectrum shows (Figures $4_{A,}$ 4_{B}), in CS NPs, the peak of 1658 cm^{-1} shifted to 1642.7 cm^{-1} and the relative intensity of this peak was reduced. In addition, the peak of C─N stretch (1374 cm^{-1}) disappeared and a new sharp peak appeared at 1382.5 cm^{-1}. The peak at 897 cm^{-1} was the result of the saccharide structure of CS (Silverstein *et al* 2005). So, it can be supposed that the TPP ions interact with the primary amino groups of the CS, resulting in the formation of cross-linked CS particles (reticulation process) (Borges *et al* 2005).

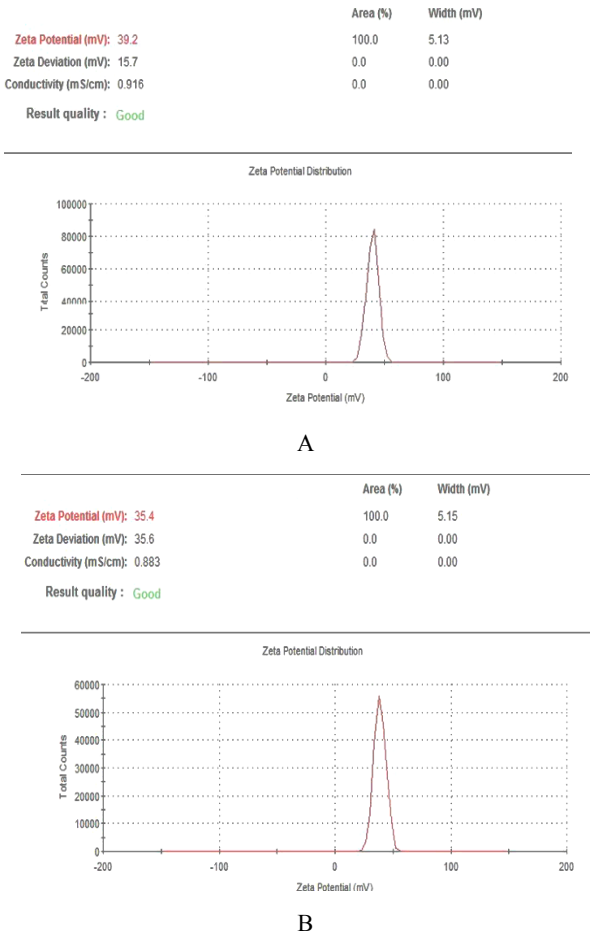
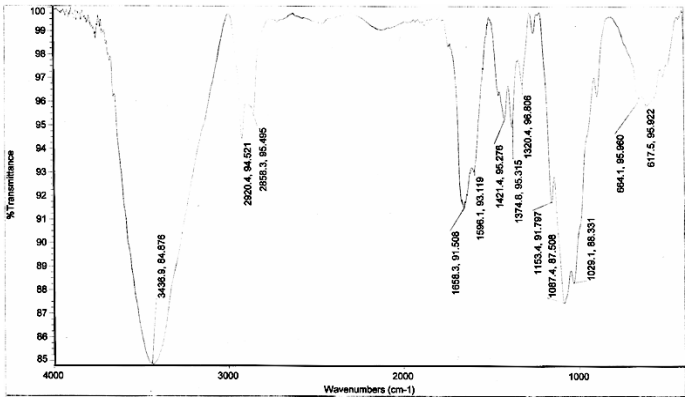


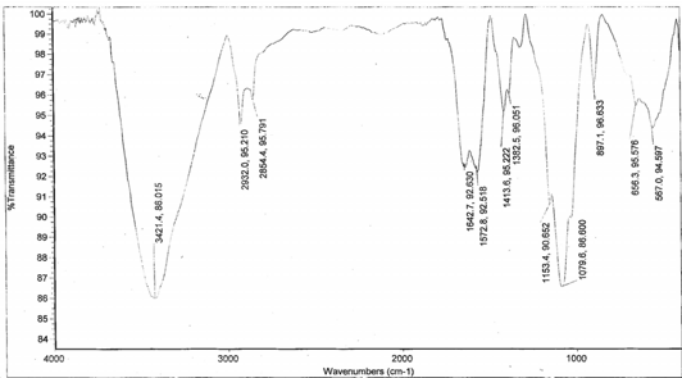
Figure 3. Z potential of nanoparticles. (A) chitosan nanoparticles . (B) *Hemiscorpius lepturus* (*HL*) venom loaded chitosan nanoparticles.

Moreover, since protein molecules are macromolecules with 3-D structure and are able to fold and unfold at different solution conditions, their interactions with long cationic chitosan and, consequently, their encapsulation can be complicated depending on 3-D conformation, electrostatic and

solution conditions. As a crosslink agent, TPP forms further hydrogen bonds with amine groups on both venom and CS molecules and results in more compact protein- CS NPs. Additional adsorption of protein molecules on the surface of the formed particles may occur in sequence leading to additional protein loading on the particles (Gan & Wang 2007). Protein adsorption and encapsulation by electrostatic interactions normally exhibit a saturation kinetics which reaches a peak value at high concentration under constant temperature.



A



B

Figure 4. FTIR spectrum of Chitosan (curve A) and Chitosan nanoparticles (curve B).

According to the observations, there is a linear relationship between AE and increasing concentrations of *HL* venom (Table 1).Therefore, it could be suggested that *HL* venom concentration used in the present study was lower than the concentration required for reaching the peak of AE. This linear relationship between AE and *HL* venom concentrations can be explained by the interactions between CS and *HL* venom and cross linked CS with TPP and *HL* venom. A study on Hemicalcin (peptide fraction of *HL* venom) indicated the net global charge +8 (Shahbazzadeh *et al* 2007). So, it seems that another factor playing a role in the above mentioned linear relationship is probably due to the simultaneous interaction of TPP with *HL* venom and CS. Reports on the effect of protein concentration on encapsulation are inconclusive and sometimes contradictory. While the findings of this work are in agreement with the studies carried out by some researchers (Berthold et al 1996, Mohammadpour dounighi *et al* 2010, Rezaei Mokarram *et al* 2006 , Avadi *et al* 2010), they are in contrast with the findings of Xu and Du (Xu & Du 2003), who reported the reversed results on BSA (Bovine serum Albumin) encapsulation at pH 6.0 and the same trend was also reported for α-Lactalbumin, cytochrome C and ribonuclease A (Somnuk *et al* 2011, Sabnis & Block 2000). Xu & Du also reported that CS effectiveness in coagulation solution solids and proteins was inversely proportional to its Mw (Xu & Du 2003). There is another study which showed that high viscosity associated with increased CS concentration hindered the encapsulation of BSA by deterring BSA molecule movement around the CS molecular chain (Vandenberg *et al* 2001). The present study showed that when the concentration of CS increased, the loading capacity increased in constant venom concentration (Table 1).

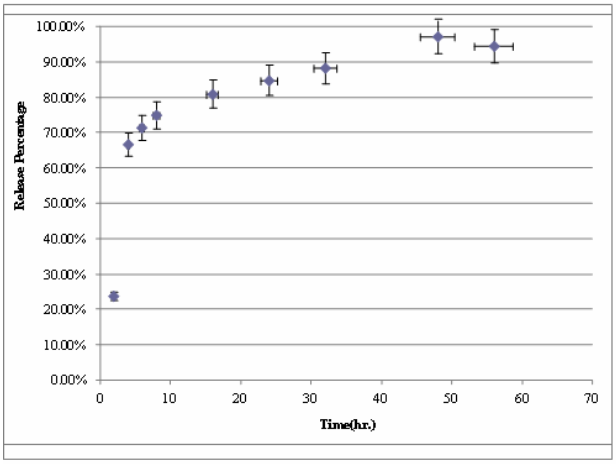


Figure 5. The *in vitro* release behavior of chitosan nanoparticles containing *Hemiscorpius lepturus* venom. (CS 2mg/ml and *HL* venom 500µg/ml), (mean ± SD, n=3).

This study revealed that there are possibilities to modulate the release rate of *HL* venom from CS NPs by adjusting the concentration of *HL* venom.

Table 1. The influence of *Hemiscorpius lepturus* venom and chitosan concentration on encapsulation efficiency and loading capacity , (mean ± SD , n=3).

Hemiscorpius lepturus Venom Concentration (µg/ml)	Chitosan Concentration (mg/ml)	%AE ± SD	%LC ± SD
21	1.5	10.70 ± 0.84	2.90 ± 0.72
	2	34.52 ± 1.04	6.22 ± 0.94
	3	40.36 ± 0.91	6.43 ± 0.76
43	1.5	18.66 ± 0.55	9.90 ± 0.86
	2	47.68 ± 1.21	12.43 ± 1.07
	3	44.72 ± 1.10	11.73 ± 0.68
64	1.5	25.78 ± 0.93	12.24 ± 0.96
	2	64.86 ± 1.05	41.58 ± 1.04
	3	61.06 ± 1.45	24.24 ± 1.08
500	1.5	68.42 ± 1.31	73.36 ± 1.14
	2	94.71 ± 2.34	89.48 ± 1.49
	3	93.13 ± 1.10	89.72 ± 0.96
750	1.5	70.98 ± 2.34	84.97 ± 1.42
	2	95.52 ± 1.08	86.88 ± 1.39
	3	96.62 ± 1.30	86.75 ± 1.23

Some reports on microspheres showed that the release process involved two different mechanisms that were the diffusion of protein molecules and degradation of polymer matrix. The burst release of protein depended on those protein molecules dispersing close to the microspheres surface, which easily diffused out in the initial incubation time (Zhou & Li 2001, Zhou *et al* 2004, Zhou *et al* 2002). From the release profile of CS NPs containing *HL* venom (Figure 5), it can be concluded that the nanoparticles with huge specific surface can adsorb HL venom; therefore, the venom can be easily released in the first few hours and the later release is due to the slow degradation of nanoparticles leading to the release of entrapped venom with a constant rate.

Currently in the antivenoms manufacturing process, the traditional adjuvants (such as water-oil emulsions) used as an antigen delivery create some problems and have only weak immunostimulating capacity. In the other hand, their preparation process is very costly and time consuming compared to CS nanoparticles. CS NPs prepared in this work showed desirable characteristics, an excellent efficiency and capacity for the loading of *HL* venom and suitable release kinetics in vitro. It is suggested that the CS NPs fabricated in the present study had the potential to be used as an alternative for traditional adjuvant systems.

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