I.A.U. Karaj Branch

Journal of Applied Chemical Research, 7, 3, 35-46 (2013)

Journal of Applied Chemical Research
www.jacr.kiau.ac.ir

Preparation, Characterization and in vitro Studies of Chitosan Nanoparticles Containing *Androctonus Crassicauda scorpion venom*

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(Received 14 Mar. 2013; Final version received 22 Jul. 2013)

Abstract

Many strategies have been developed to improve vaccine delivery in the past decade. The aim of the current study was to develop a nanoparticulate system based on ionic gelation between chitosan and tripolyphosphate in order to load *Androctonus Crassicauda* scorpion venom. The best formulation was selected according to the highest association efficiency, loading capacity, optimum particle size and zeta potential. Venom release studies were carried out on the optimum formulation F3. The highest association efficiency (77%) and loading capacity (48%) were obtained from formulation containing chitosan (2.0mg/ml), TPP (1mg/ml) and scorpion venom concentration of 250µg/ml. The average size of nanoparticles in optimum formulation was about 230 nm with a polydispersity index of 0.479 and a positive zeta potential value. The FTIR results also confirmed the binding of TPP and chitosan with the venom peak present in the nanoparticle formulation. The *in vivo* release of venom from nanoparticles at pH 7.2 showed an initial burst release of about 69% in the first 24 hours followed by a sustained and much slower release in the next 96 hours. The result suggested that this nanoparticulate system maybe suitable as an antigen carrier with anti- venom activity for further *in vivo* studies.

***Keywords**: Chitosan, Ionic gelation, Scorpion venom, Androctonus Crassicauda, Nanoparticle characterization, Release studies.*

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Introduction

Scorpion stings cause a wide range of conditions, from severe local skin reactions to neurologic, respiratory and cardiovascular collapses. The Androctonus Crassicauda scorpion, also known as fat-tailed scorpion, is widely found in Saudi Arabia and neighboring countries. The scorpion is abundant in Iran, Iraq, Turkey, Egypt Sudan, Syria, Jordan, Morocco and Saudi Arabia [1-4]. Scorpionism is a known significant problem of medical and social importance in many North African countries and in the Middle East, in Mexico and Brazil [5]. Among 1500 species described, venoms of 50 species are dangerous for humans and most of these species belong to Buthus, Parabuthus, Mesobuthus, Tityus, Leiurus, Androctonus, Centruroides genuses of Buthidae family [6].

Androctonus are known to belong to the Buthidae family and proven dangerous to humans; documented cases of severe intoxication by this scorpion requiring serum therapy were reported [7]. Although almost nothing is known about their venom components, a substantial amount of knowledge has been accumulated during the last two decades on the isolation and characterization of peptides and genes from scorpion venoms.

Recently, many strategies have been developed to enhance protein delivery [8, 9]. Among these approaches, nanoparticulate systems have attracted special interest for three main reasons. First, nanoparticles are able to protect active agents from degradation [10]. They can improve transmucosal transport of drugs and transcytosis by M cells [11]. The particulate systems can provide controlled-release properties for encapsulated drugs [12]. Chitosan, a (1,4)-2-amino-2-deoxy-β-D-glucan, is a deacetylated form of chitin, an abundant polysaccharide present in crustacean shells. Chitosan is a nontoxic polymer that has been used in biomedical fields in the forms of sutures, wound coverings, and as artificial skin [13]. Chitosan is a biodegradable and biocompatible polymer that due to its cationic nature has good mucoadhesive and membrane permeability–enhancing properties [14]. Hence, chitosan has been extensively investigated for its potential as an absorption enhancer across intestinal epithelium for peptides and proteins [14]. Chitosan is insoluble under alkaline and neutral conditions but is able to react with inorganic and organic acids such as hydrochloric acids, acetic acid, and glutamic acid under acidic conditions. The mucoadhesive property of chitosan is mediated by its ability to spread over the mucous layer and additionally through its positive ionic interactions with the negative charges of the mucus or of the cell surface membranes [15].

Due to the presence of amine groups, chitosan has many applications in pharmaceutical industry. Chitosan has higher positive charge than other natural polymers; therefore, it is used extensively in drug delivery systems [16]. The most applied method for preparing chitosan

nanoparticles are bottom-up approaches which are a result of a self–assembling or cross linking interactions. In these nanostructures, drugs are entrapped inside nanoparticles or can be attached to the matrix [17].

Ionotropic gelation between chitosan, a polycation, and sodium tripolyphosphate (TPP), a polyanion, leads to the formation of colloidal systems which can range from approximately 100nm to 2000nm. Depending on the conditions, particles with different properties can be obtained including differences in size, zeta potential and loading characteristics. The advantage of this method is attributed to the mild conditions without the application of harmful organic solvent, heat or vigorous agitation that are harmful to sensitive proteins. Moreover, it could efficiently retain the bioactivity of macromolecules (such as DNA, proteins, etc) during preparation [18]. The electrostatic interaction is particularly suited for the incorporation of biopharmaceuticals for two reasons: first, the formation process is solely based on the electrostatic interaction of oppositely charged polymers; hence, no chemical modification is needed. Secondly, the incorporation can be achieved in aqueous, physiological conditions [19, 20]. The aim of the current study was to develop a novel vaccine delivery system in order to prepare a new type of antigen carrier and improve the anti-venom effects by nanotechnology science. Moreover, properties of chitosan nanoparticles containing venom such as association efficiency, loading capacity, particle size, polydispersity index (PI) and in vitro release studies were evaluated.

Experimental

Materials

Chitosan (95% deacetylated with viscosity of 1% w/v solution, 30 cp) was purchased from Primex (Siglufjordur, Iceland). Sodium Tripolyphosphate (STPP) and acetic acid were purchased from Sigma and from Merck Co. (Germany), respectively. Androctonus Crassicauda venom was provided as a freeze-dried powder by Razi Vaccine and Sera Research Institute (Karaj, Iran). Coomassie brilliant blue G250 was purchased from Sigma Chemical Co. (USA). The other materials were of pharmaceutical and analytical grades and were used as received.

Methods

Preparation of chitosan nanoparticles containing venom

The *Androctonus Crassicauda* venom nanoparticles were prepared by ionic gelation between positively charged chitosan and negatively charged TPP. Initially, known amounts of chitosan were dissolved in 1.0% acetic acid aqueous solution to obtain concentrations of 1.0, 1.5, 2.0, 2.5 and 3.0 mg/ml under stirring at room temperature. Subsequently, TPP solution (1.0 mg/ml) was

prepared in double-distilled water. A fixed amount of *Androctonus Crassicauda* venom (250µg/ml) was added to TPP solution under magnetic stirring at room temperature (Table1). Chitosan nanoparticles were spontaneously fabricated with the drop-wise addition of TPP solution containing venom to chitosan solution under constant magnetic stirring (IKA, RETB, Germany, 800 rpm, 15 minute) at room temperature. The nanoparticles were separated by centrifugation at 15000 rpm at 4°C for 30 min (sigma 3K30, Osterode, Germany), freeze-dried using primary drying at -40°C and 0.050 mbar for 30 hour and then secondary drying at 0.021 mbar at -55°C for 18 hour and consequently stored at 5.0±3°C. The weight of freeze-dried nanoparticles was also measured (AND, HR-200 Japan, sensitivity 0.1mg). Blank nanoparticles (free of venom) were also prepared and used for comparative reasons.

Characterics of chitosan nanoparticles containing venom

The size of the scorpion venom nanoparticles were measured with a Malvern zeta sizer (Malvern Instruments, Worcestershire, United Kingdom). The particle size distribution is reported as a PDI. The range for the PDI is from 0 to 1; values close to zero indicate a homogeneous dispersion and those greater than 0.5 indicate high heterogeneity. The samples were placed in the analyzer chamber and readings were performed at 25°C with a detected angle of 90 degrees. The zeta potential of nanoparticles was measured with a zeta sizer (Malvern,UK). The samples were diluted with double-distilled water. Each sample was measured three times, and values were presented as the mean standard deviation (SD). Transmission electron microscopy (TEM) was used to observe the morphology of chitosan nanoparticles (Zeiss, em10C, 80 kV, Germany). The structural features of nanoparticles were estimated by FTIR (FTIR- 410 Jasco Colchester, United Kingdom), using KBr pellets.

Venom association efficiency and loading capacity

The association efficiency (AE) was determined indirectly after separation of venom containing nanoparticles from the aqueous medium containing non-associated venom. The amount of the free venom was measured by the Bradford protein spectrophotometric method at 595 nm [21]. The association efficiency of venom nanoparticles and venom loading capacity (LC) were calculated according to the following equations:

$$AE\% = [(A-B)/A] \times 100$$

$$LC\% = [(A-B)/C] \times 100$$

Where A is the total amount of venom, B is the free amount of venom and C is the weight of nanoparticles containing venom.

Release studies

Venom release from nanoparticles was

performed at phosphate buffer solution (PBS), pH 7.2. Briefly, freeze-dried nanoparticles (containing 2.0 mg venom) were placed in a 25-mL test tube and set into dissolution Erweka apparatus (DT6; Heusentamn, Germany). The amount of nanoparticles in the release medium was adjusted for sink condition. The temperature and rotation were set at 37°C and 100 rpm, respectively. The samples were taken at predetermined time intervals 2, 6, 10, 24, 34, 48, 72 and 96 hour. The amount of venom released in the dissolution media was measured by the Bradford protein assay.

Statistical Analysis

The data was analyzed using SPSS (V.19.0.0) and the Tuckey HSD; Scheffe and LSD Post Hocs were used for statistical comparison. The significance level was considered as 0.05 in all cases. The Levene Statistic test showed homogeneity of variances in all cases (P>0.05).

Results and discussion

Preparing chitosan nanoparticles containing venom

The aim of this study was to investigate the influence of variable chitosan concentrations in the preparation of venom nanoparticles using the ionic gelation method. According to Table1, various formulations (F1 to F5) with different physicochemical properties such as particle size, PDI and zeta potential were prepared (data presented in Table 2). The results of Loading Capacity showed the highest value for formulation F3 and the lowest value for F1 formulation; although not a significant difference in loading capacity between formulations F3 and F4 (P<0.05) were observed.

Table 1. The concentrations of chitosan, TPP and venom in different formulations.

Formulation No.	Chitosan Concentration (mg/ml)	TPP Concentration (mg/ml)	Venom Concentration (µg/ml)
Blank	1.0	1.0	-----
F1	1.0	1.0	250
F2	1.5	1.0	250
F3	2.0	1.0	250
F4	2.5	1.0	250
F5	3.0	1.0	250

Table 2. Characteristics of the nanoparticles containing venom for different formulations F1 to F5 (n=3).

Formulation No.	Mean diameter, (nm)	Polydispersity index	Mean Zeta potential (mV)
Blank	267	0.401	37.7
F1	134	0.400	13.7
F2	228	0.442	34.0
F3	230	0.479	35.2
F4	311	0.463	35.9
F5	1260	0.829	20.0

In these formulations the amount of both venom (250µg/ml) and TPP concentration (1 mg/ml) were kept constant. With chitosan concentration of 3.0 mg/ml, the obtained nanoparticles were shown to have particle size of approximately 1260nm with PDI values of 0.829 that were not acceptable. Therefore, concentrations 1.0, 1.5, 2.0 and 2.5 mg/ml of chitosan were selected for further investigations. It should be noted that variables including rate of mixing (800 rpm), the rate of addition of TPP solution to chitosan solution and the time of mixing (15-20 min) were kept constant. Blank chitosan nanoparticles were prepared before complementary studies and formulations F1-F4 were compared to blank nanoparticles (Table 2). The mean particle size and PDI for blank nanoparticles obtained were 267 nm and 0.401, respectively. When the chitosan nanoparticles were prepared with concentration of 1 mg/ml the mean of particle size, PDI and zeta potential of major peak were 134 nm, 0.400 and 13.7 mV, respectively. The particle size and zeta potential in formulation F2 for chitosan nanoparticles with concentration of 1.5 mg/ml were 228 nm and 34.0 mV, respectively. It seems that the particle size was higher compared to the formulation F1, possibly due to an increase in chitosan concentration. The high amount of chitosan in formulation F2 compared to F1 resulted in an increase in positive density and viscosity of solution, so the resistance of aqueous medium to dispersion was increased and consequently caused a slow diffusion of TPP solution toward the chitosan solution and increment of particle size.

According to Table 2, the zeta potential in formulation F2 was higher compared to formulation F1. This may be related to stronger positive charges of the amino group of chitosan at high level in the experiment. The PDI was another factor that represented the dispersion homogeneity. The PDI for all the formulations as shown in Table 2 was lower than 0.5, which indicates a relative homogeneous dispersion. Increasing chitosan concentration in formulation F3, resulted in chitosan nanoparticles with higher particle size. The mean particle size of formulation F3 (230 nm) was higher than formulation F2. The residual amine groups would possibly be responsible for the positive zeta potential (35.2 mV). In the last formulation (F4) the particle size and zeta potential were increased to 311 nm and 35.9 mV, respectively; probably due to the residual amine groups present (Table.2). Moreover, all the nanoparticle formulations (F1-F4) were positively charged, but the values for F3 and F4 were higher than those of the other formulations, which may be related to stronger positive charges of the amino group of chitosan at high level in the experiment. This could be related to the type of particle formation mechanism in ionic gelation, where positively charged amine groups of chitosan

were neutralized by their interaction with negatively charged TPP solution and the residual positive amine group was responsible for final positive charged nanoparticles.

Table 3. AE and LC of nanoparticles containing venom for different formulations F1 to F4 (n=3).

Formulation No.	AE (%) mean±SD	LC (%) mean±SD
F1	69±5	26±4
F2	72±8	37±6
F3	77±6	48±5
F4	74±7	41±6

As shown in Table 3, the optimum AE and LC were obtained for four formulations F1 to F4. The amount of chitosan in formulation F3 and F4 were at a higher level (Table 3) and caused an increase in AE, possibly due to the higher ability of ionic gel formation compared to formulations F1 (lower level of chitosan) or F2; however, the difference AE in formulations F3 and F4, is not significant. This may be due to the maximum capacity of trapping venom in nanoparticle system which confirmed in formulation by higher concentration of chitosan (3 mg/ml in formulation F5) compared to formulation F4.

In this study, the addition of chitosan solution to TPP solution containing venom was also investigated. It was shown that although nanoparticles were formed, after 1-2 hour they tend to aggregate due to an increase in particle size and consequently resulting in precipitation (data has not been shown). Indeed, when the chitosan solution was added to TPP solution containing venom, the positively charged chitosan polymer rapidly interacted with less negatively charged sites present on TPP and eventually aggregation was observed. Hence, the procedure of adding TPP solution containing venom to chitosan solution was selected and used. However, the studies have shown that when TPP solution was added to the chitosan solution, the nanoparticles were formed rapidly and the venom was surrounded by a polymeric network due to interactions between two polyelectrolyte agents.

According to Table 3, the loading capacity for none of the formulations was high which may be due to the presence of venom on the surface of nanoparticles which were in direct contact with the external phase. According to physical characteristics of the nanoparticles obtained as well as the percent of AE and LC (Tables 2 and 3), formulation F3 was selected as the optimum formulation and used for release studies.

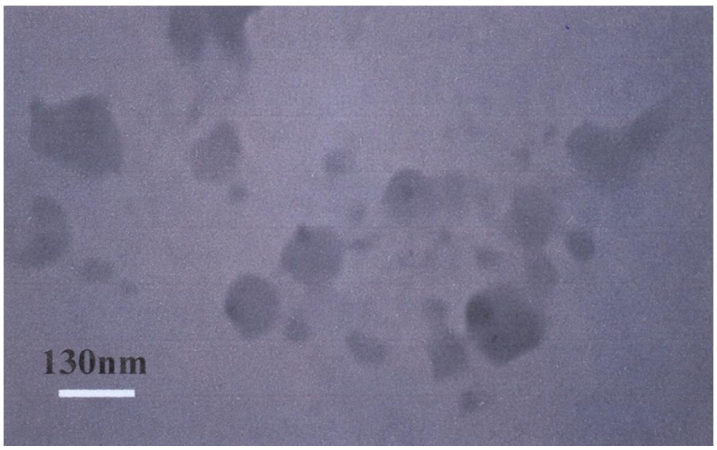

Figure 1. TEM micrographs of chitosan nanoparticle containing venom for formulation F3.

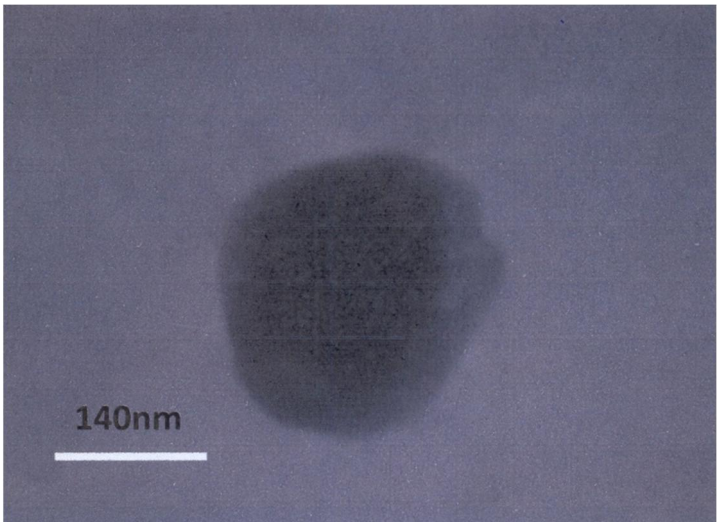

Figure 2. TEM micrographs of single nanoparticle for formulation F3.

TEM images have shown the morphological and surface appearance of nanoparticles. Chitosan nanoparticles containing venom in formulation F3 has shown in Figure 1. As was shown in this figure, nanoparticles have rod like, nearly spherical shape with size range about 150-300 nm. A TEM image of separate nanoparticle has shown in Figure 2. TEM picture has confirmed the data obtained from nanoparticle size measurement by particle size analyzer. According to Figures 1 and 2, the positive charges on the surfaces of nanoparticles could prevent the agglomeration process that corresponds nicely with the results of zeta potential observations.

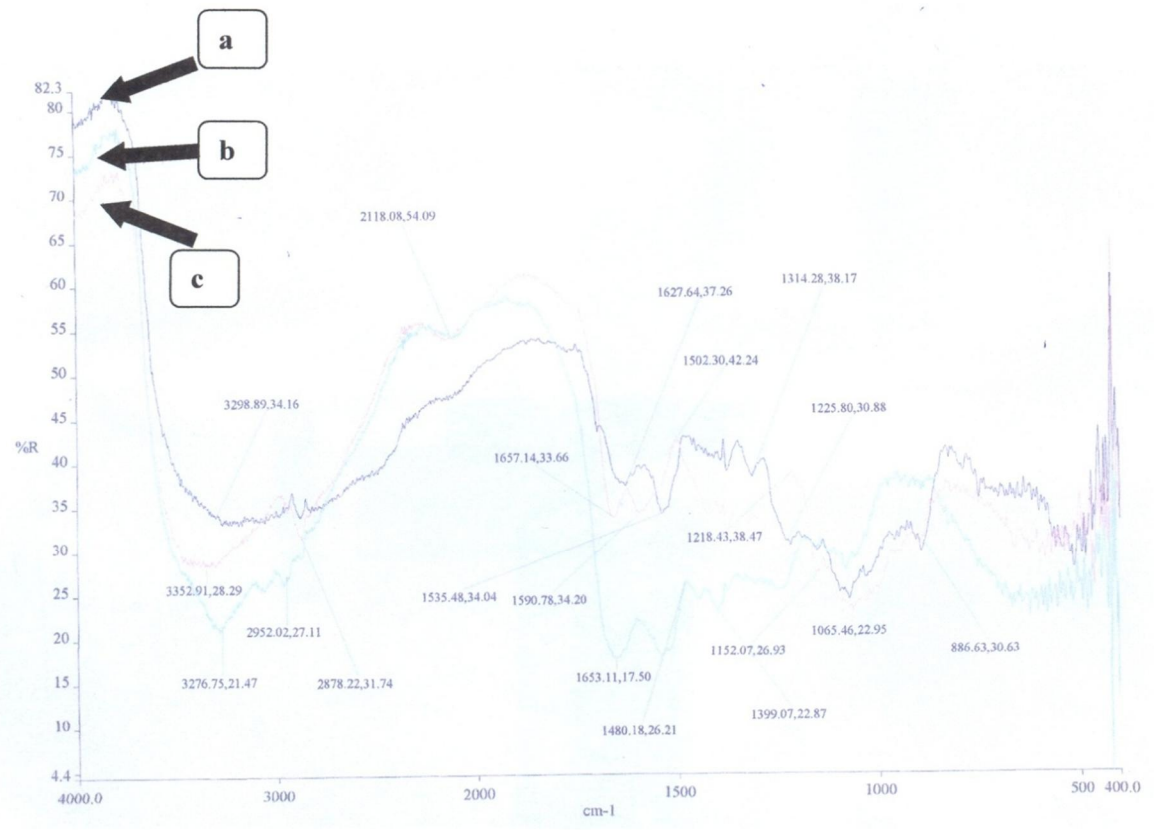

Figure 3. FTIR spectrum of chitosan (a), Venom (b) and chitosan nanoparticles containing venom (c).

The FTIR spectrums for chitosan, venom and chitosan nanoparticles are presented in Figure 3. The data presents a strong wide peak about 3000-3500 cm^{-1} that is related to hydrogen-bounded O-H stretching vibration (Figure 3a). Primary amine N-H stretching vibration has the same absorption in this region that overlap with O-H stretching vibration peak. The peak of stretching vibration for C-N in primary amine is observed in 1314 cm^{-1}. The peaks at 1359 cm^{-1} and 1638 cm^{-1} belong to N-H bending vibration in primary amine and carbonyl group stretching vibration in amide type II, respectively. An asymmetric stretching vibration peak of C-O-C is observed in 1150 cm^{-1} (Figure 3a). FTIR spectrum of chitosan nanoparticles has shown in Figure 3c. The peak of 3261 cm^{-1} has shifted to 3321 cm^{-1} and became less wide due to hydrogen bonding formation. The peaks in N-H bending vibration and carbonyl stretch in amide type II have shifted to 1590 cm^{-1} and 1657 cm^{-1}, respectively. Moreover, according to interaction of positive charge of chitosan with negative charge of TPP and cross-linked chitosan, a peak is obtained for P=O in 1152 cm-1. FTIR spectrum of venom has shown in Figure 3b. The peaks at 2118 cm^{-1} and 2878 cm^{-1} for venom have the same peaks in chitosan nanoparticles containing venom indicating of venom presence in nanoparticles.

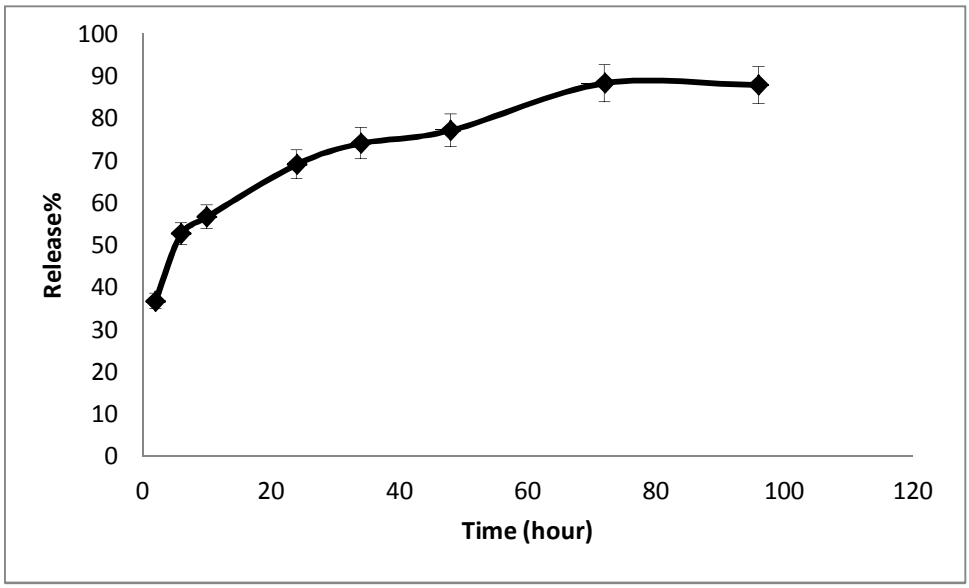

Figure 4. In vitro release profile of scorpion venom from chitosan nanoparticles in PBS, pH 7.2 medium (n=3).

Venom release studies from chitosan nanoparticles were carried out using phosphate buffer solution pH 7.2 [22] and protein detection has done by Bradford method. Figure 4 has shown the profile of venom release from nanoparticles. According to this profile a burst effect of venom release was observed within 24 hour. This may be related to venom macromolecules that were loosely bound to the surface of chitosan nanoparticles. Moreover, venom macromolecules has hydrophilic property that resulted in diffusion of venom is located on the nanoparticle surface into the aqueous medium [23]. The residue section of release profile has shown approximately constant release rate, this slow release of venom may be related to venom protein entrapment. Furthermore, chitosan nanoparticles produce a polymeric network that resulted in the entrapment of the venom protein in polymeric matrix. Moreover, it seems that the interaction between two oppositely charged polymers could obtain a strong matrix polymer network that caused venom trapping in this matrix network and finally decreased venom release in PBS at pH 7.2 medium. Therefore, in the first stage, the medium (PBS) may be diffused in polymeric network and in next stage the polymeric chains undergo relaxation and swelling behavior and finally venom is dissolved in PBS and diffuses into the medium.

Conclusions

Currently various types of adjuvant such as solution, suspension and emulsion systems are used for immunization against scorpion venom as an antigen delivery system. The major problems with these systems are their low immunostimulating ability, their costly

preparation and time consuming processes.

In this study, chitosan nanoparticles loaded with Androctonus Crassicauda scorpion venom were prepared based on ionotropic gelation method, using TPP as cross linker. The physicochemical properties of these nanoparticles were investigated. The nanoparticles were obtained with low molecular weight chitosan in concentration of 2.0 mg/ml, venom concentration of 250 µg/ml, and TPP concentration of 1.0mg/ml. Optimum nanoparticles with size range of 150-300 nm (mean size about 230 nm) with loading capacity of 48%, encapsulation efficiency of 77% and acceptable PDI (0.479) were obtained. Hydrophilic nanoparticles based on chitosan are receiving increased interest as they could control the rate of drug release, prolong the duration of the therapeutic effect, and deliver the drug to specific sites in the body. It was shown that the release of venom from nanoparticles was obtained with more than one mechanism (possibly diffusion, dissolution, and relaxation of the polymer chains). Results have indicated a small size, positive charge, and suitable AE for nanoparticles; therefore, the chitosan nanoparticles containing scorpion venom may be used as alternative candidates for traditional adjuvant systems. The obtained data showed that all variables should be taken into account during the formulation of such a system.

Acknowledgements

We would like to extend our thanks to Dr. H. Mir-Mohammad Sadeghi and Dr. M. Alimian for their kind support throughout this study. Special thanks goes to the R&D personnel of Hakim Pharmaceutical company especially Mr. Solukaneh for his great assistance in this project.

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