ORIGINAL PAPER

The Journal of Venomous Animals and Toxins including Tropical Diseases
ISSN 1678-9199 | 2012 | volume 18 | issue 1 | pages 44-52

Preparation and *in vitro* characterization of chitosan nanoparticles containing *Mesobuthus eupeus* scorpion venom as an antigen delivery system

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Abstract: Hydrophilic nanoparticles have been widely investigated in recent years as delivery systems for therapeutic macromolecules such as antigens. In the present study *Mesobuthus eupeus* venom-loaded chitosan nanoparticles were prepared via ionic gelation of tripolyphosphate (TPP) and chitosan. The optimum encapsulation efficiency (91.1%) and loading capacity (76.3%) were obtained by a chitosan concentration of 2 mg/mL, chitosan-to-TPP mass ratio of 2 and *M. eupeus* venom concentration of 500 µg/mL. The average nanoparticle size at optimum conditions was determined by Zetasizer (Malvern Instruments, UK). The nanoparticle size was about 370 nm (polydispersity index: 0.429) while the zeta potential was positive. Transmission electron microscope (TEM) imaging showed a spherical, smooth and almost homogenous structure for nanoparticles. Fourier transform infrared (FTIR) spectroscopy confirmed tripolyphosphoric groups of TPP linked with ammonium groups of chitosan in the nanoparticles. The *in vitro* release of nanoparticles showed an initial burst release of approximately 60% in the first ten hours, followed by a slow and much reduced additional release for about 60 hours. It is suggested that the chitosan nanoparticles fabricated in our study may provide a suitable alternative to traditional adjuvant systems.

Key words: nanoparticle, chitosan, tripolyphosphate, venom, antigen delivery system, *Mesobuthus eupeus*.

INTRODUCTION

Today, nanotechnology is found in a wide range of applications in the pharmaceutical industry (1). Due to new advances in nanotechnology, it is now possible to produce drug nanoparticles that can be utilized in a variety of innovative ways (2).

Injectable nanoparticulate carriers have important potential applications even though conventional carriers can generally be used to reduce the number of administration doses and improve delivery efficiency while decreasing the adverse effects of drug toxicity. In order to achieve this aim, monodispersed biodegradable nanospheres were developed that could be freeze-dried and easily redispersed, without additives, in aqueous solutions. These drug carriers have been applied in different pharmacological fields (3-5).

One area of interest involves the interactions between nanoparticles and the components of the immune system. Nanoparticles can be designed to either avoid immune system recognition or specifically reduce or enhance the immune responses. Nanoparticle-mediated stimulation and suppression of the immune system can be explained by the fact that manipulation of particle physicochemical characteristics can influence its interaction with immune cells to obtain desirable immunomodulation and avoid undesirable immunotoxicity (6). Moreover, the potential of nanoparticles as an antigen delivery system has been shown in numerous studies (7, 8).

Both synthetic and natural polymers were studied with the aim of forming nanoparticles (9). However, among the variety of polymers that were used for drug-loaded nanoparticles,

chitosan has received great attention in both the medical and pharmaceutical fields (10). Chitosan, a biodegradable and biocompatible polymer, is a modified natural carbohydrate and the second most abundant polysaccharide in nature. It can be synthesized by the partial N-deacetylation of chitin, a natural biopolymer derived from crustacean shells such as crabs, shrimps and lobsters (11). It consists of repeating units of glucosamine and N-acetyl-glucosamine, the proportions of which determine the degree of deacetylation of the polymer (12). Chitosan is available in a wide range of molecular weights and deacetylation degrees. Due to its characteristics, chitosan has gained increasing attention in the pharmaceutical field.

In addition, chitosan presents mucoadhesive, immunostimulating, antimicrobial and wound-healing properties (13-15). Moreover, it has been regarded as a promising polymer for the formulation of vaccine delivery systems. On the other hand, the evaluation of chitosan as an adjuvant for parenteral vaccination studies was reported together with the results of intranasal or oral vaccination studies, making the possible value of chitosan as an adjuvant for parenteral routes less noticeable in the scientific literature. Generally, the design and development of safe novel adjuvants is necessary not only for overcoming the more challenging environment of the mucosal surfaces, but also for vaccination by injection routes, to maximize the efficacy of new or already available vaccines. In the last few years this idea became even more important since newer generations of antigens are predominantly purified recombinant proteins, which often present poor immunogenicity (16).

Chitosan nanoparticles can easily be prepared by the ionic gelation method using TPP as a crosslinking agent. The advantage of this method was attributed to its mild conditions achieved without applying harmful organic solvent, heat or vigorous agitation that are damaging to sensitive proteins. Moreover, it could efficiently retain the bioactivity of macromolecules (such as DNA, proteins, etc.) during preparation (17). It has been reported that chitosan nanoparticles have an excellent capacity for associating proteins (18). CS nanoparticles are widely investigated for delivery of polypeptides such as tetanus toxoid, diphtheria toxoid and snake venom (19-21).

Scorpion venom is rich in various polypeptides with diverse physiological and pharmacological activities (22-24).

The major goal of the current study was to develop a novel antigen delivery system in order to promote the anti-venom manufacturing by improving the hyper-immunization of animals. For this purpose, an effort is being made to prepare new biodegradable nanoparticles for loading of *Mesobuthus eupeus* scorpion venom and to evaluate their potential as an antigen delivery system.

MATERIALS AND METHODS

Materials

Low molecular weight chitosan (48 kDa) derived from shrimp shells (*Pandalus borealis*), was purchased from Primex Co (Iceland).

The molecular weight of polymer was determined by viscometry (the degree of deacetylation claimed by the supplier was 95%). Sodium tripolyphosphate (STPP) and coomassie blue G250 were supplied by Sigma (USA). Phosphoric acid (85%), acetic acid and absolute ethanol were purchased from Merck (Germany).

M. eupeus venom was provided in the form of a lyophilized powder by the Razi Vaccine and Serum Research Institute (Karaj, Iran). All other reagents utilized in this study were of analytical grade.

Preparation of Chitosan Nanoparticles and Venom-Loaded Nanoparticles

Chitosan nanoparticles were synthesized via the ionotropic gelation (25-27) of chitosan with TPP anions. Chitosan was dissolved in acetic aqueous solution at various concentrations (1, 2, 3 mg/mL). The concentration of acetic acid in aqueous solution was 1.5 time higher than that of chitosan (28). The TPP solution (1 mg/mL) was prepared by double-distilled water. Chitosan nanoparticles were spontaneously fabricated with the dropwise addition of 5 mL of the chitosan solution to 2 mL of TPP solution under magnetic stirring (1000 rpm, 1 hour) at room temperature. The opalescent suspension was formed under the same abovementioned conditions. The nanoparticles were separated by centrifugation at 20,000 g and 14°C for 30 minutes, freeze-dried and stored at 5 ± 3°C. The weights of freeze-dried nanoparticles were also measured.

Venom-loaded nanoparticles were formed by the addition of chitosan solution to TPP solution containing different concentrations of venom. In the present work the effects of venom concentrations (50, 75, 100, 200, 300 and 500 µg/mL) and chitosan concentrations (1, 2, 3 mg/mL) on nanoparticle's characteristics have been studied. In order to study one of the abovementioned parameter, the other parameters remained constant.

Characterization of Chitosan Nanoparticles

The morphological characteristics of nanoparticles were investigated by transmission electron microscope (TEM) (Philips 400®, 80 kV, The Netherlands). The samples were immobilized on copper grids and dried at room temperature. Then they were stained with phosphate tungsten acid and examined by TEM. The particle size, size distribution [polydispersity index (PDI)] and zeta potential of particles were measured by Zetasizer (Malvern Instruments, UK), based on the dynamic light scattering (DLS) technique. The structural features of nanoparticles were estimated by FTIR (Fourier transform infrared) (FTIR- 410® Jasco Colchester, United Kingdom), using KBr pellets.

Determination of Venom Encapsulation Efficiency and Loading Capacity of Nanoparticles

Venom-loaded nanoparticles were separated from aqueous suspension by centrifugation at 20,000 g and 14°C for 30 minutes. The supernatant was collected and protein content (free venom) in supernatant was determined by the Bradford protein assay spectrophotometric method at 595 nm (29, 30).

The venom encapsulation efficiency (*AE*) and loading capacity (*LC*) of nanoparticles were calculated as follows:

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

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

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

In vitro Release Study

The particular amount of venom-loaded chitosan nanoparticles was suspended in separate tubes containing equal volumes of 0.2 mol/L PBS solution (pH 7.4) and incubated by shaking at 37°C and 600 rpm. At appropriate time intervals (1, 2, 4, 6, 10, 22, 34, 48, 72 hours) one tube was removed and the sample was centrifuged at 20,000 g and 14°C for 30 minutes. The amount of venom released in the supernatant was measured.

RESULTS AND DISCUSSION

Physicochemical Characterization of Nanoparticles

In the present study, TEM images have shown the morphological properties and surface appearance of nanoparticles. The nanoparticles have nearly spherical shape, smooth surface and size range of about 150-350 nm (Figure 1).

The respective average diameters, measured by Zetasizer, of chitosan nanoparticles and venom-loaded nanoparticles were approximately 260 nm and 370 nm. The PDI value of chitosan nanoparticles was 0.219 while that of venom-loaded chitosan nanoparticles was 0.429, thus indicating a narrow and favorable particle size distribution (PDI < 0.5) (Table 1).

In present study the results obtained by Zetasizer revealed that the venom-loaded nanoparticles are larger than the chitosan-TPP ones, possibly due to the high molecular weight and large size of the venom protein molecules, venom surface adsorption during incubation time and negligible elevation of viscosity by venom in the loading process (31). Zeta potential of venom loaded chitosan nanoparticles can greatly influence their stability in suspension by means of electrostatic repulsion between the particles (31). Our results demonstrated respective zeta potentials of chitosan and venom-loaded nanoparticles of 50.3 and 44.1 mV. These results showed that the venom loading leads to a minor reduction of the particle's zeta potential. It is supposed that the venom engagement with long chain chitosan molecules is not uniform.

Chitosan molecules are likely to adopt a diffuse conformation in the solution because of electrostatic repulsion force existing between amine groups along the molecular chain. The carboxyl groups on the surface of a large protein molecule may form hydrogen bonds with amine groups at certain sites along the chitosan chain, but still maintain a compact 3D structure without diffusing in the relatively acidic solution so as to keep an inner hydrophobic core. Therefore, protein molecule attachment did not sufficiently suppress the positive surface charge of chitosan

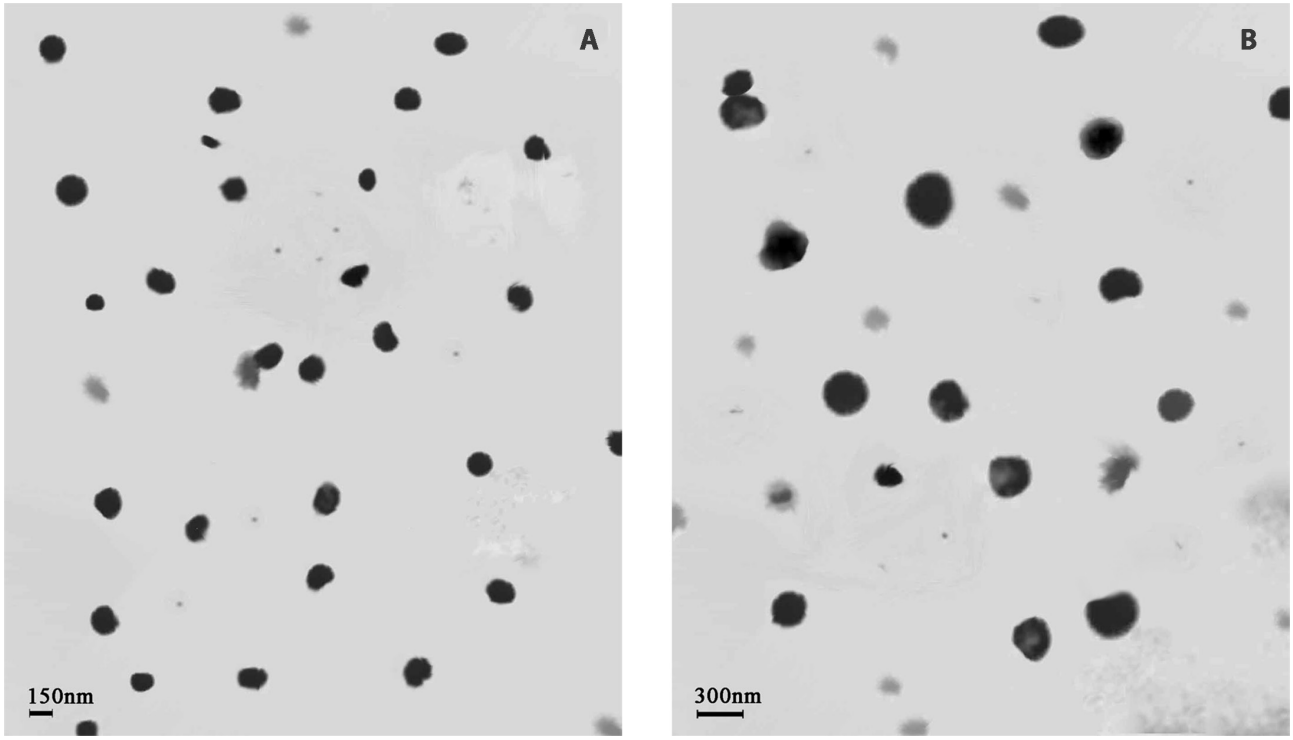

Figure 1. (**A**) TEM image of chitosan-TPP nanoparticles and (**B**) *M. eupeus* venom-loaded chitosan-TPP nanoparticles (chitosan: 48 kDa, 2 mg/mL; *M. eupeus* venom 500 µg/mL).

Table 1. Particle size, polydispersity index and zeta potential of low molecular weight chitosan nanoparticles and venom-loaded chitosan nanoparticles

Chitosan concentration (mg/mL)	Particle size (nm)		Polydispersity index [$(\mu_2)/\Gamma^2$]		Zeta potential (mV)	
	Without venom loading	Venom-loaded nanoparticles	Without venom loading	Venom-loaded nanoparticles	Without venom loading	Venom-loaded nanoparticles
1.0	132 ± 23.4	ND	0.581	ND	54.3 ± 0.6	ND
2.0	260 ± 32.1	370 ± 34.7	0.219	0.429	50.3 ± 2.2	44.1 ± 0.8
3.0	300 ± 17.6	ND	0.170	ND	47.2 ± 1.1	ND

[a] Particle preparation conditions: venom concentration 500 µg/mL, TPP concentration 1 mg/mL, T: 25 ± 2°C. ND: not determined (the physicochemical characteristics of nanoparticles prepared with chitosan concentrations 1 and 2 mg/mL is not suitable for further study); data shown are the mean ± standard deviation (n = 3).

molecules. There could still be a high proportion of amine group on the chitosan chain which remains unoccupied (31).

The ability of the ionic gelation process to form venom-loaded chitosan nanoparticles was assessed by employing FTIR to determine venom-chitosan interactions. The FTIR spectra of chitosan matrix, chitosan nanoparticles and venom loaded chitosan nanoparticles are shown in Figure 2. In the chitosan spectra, the strong and wide peak in the 3500-3300 area is attributed to hydrogen-bonded O-H stretching vibration. The peaks of N-H stretching from primary amine and type II amide are overlapped in the same region (32). The peak for asymmetric stretch of C-O-C is found at around 1150 cm^{-1} and the peak at 1317 cm^{-1} belongs to the C-N stretching vibration of type I amine. In chitosan-TPP nanoparticles the tip of the peak of 3438 cm^{-1} has a shift to 3320 cm^{-1} and becomes wider with increased relative

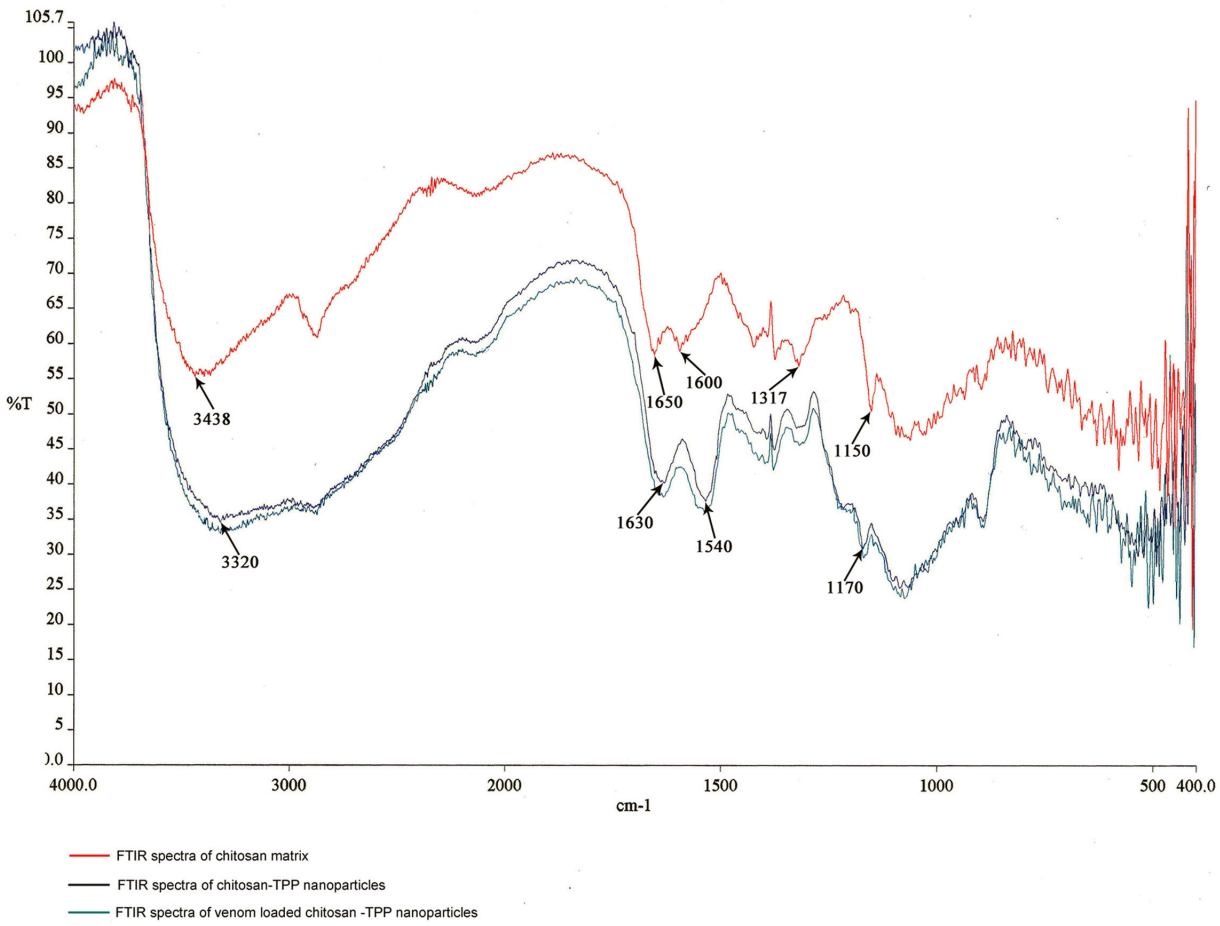

Figure 2. FTIR of chitosan, chitosan-TPP nanoparticles and *M. eupeus* venom-loaded chitosan-TPP nanoparticles.

intensity indicating an enhancement of hydrogen bonding. In nanoparticles the peaks for N-H bending vibration of amine I at 1600 cm^{-1} and the amide II carbonyl stretch at 1650cm^{-1} shifted to 1540 cm^{-1} and 1630 cm^{-1}, respectively. The crosslinked chitosan also show a P=O peak at 1170 cm^{-1}. These results have been attributed to the linkage between phosphoric and ammonium ion. So we conclude that the tripolyphosphoric groups of TPP are linked with ammonium groups of chitosan. The inter- and intra-molecular actions are enhanced in chitosan nanoparticles.

Effect of Chitosan Concentration

In the present study the effect of different chitosan concentrations (1, 2, 3 mg/mL) on nanoparticle formation was evaluated by Zetasizer. Our results showed that by increasing the chitosan concentration from 1 to 3 mg/mL at a constant TPP concentration (1 mg/mL), the size of nanoparticles increases (Table 1).

The PDI value of nanoparticles with chitosan concentration of 1 mg/mL was not within the acceptable range, as shown by the formation of aggregates with large diameters. The PDI value of particles was more favorable at the chitosan concentration of 3 mg/mL than 2 mg/mL. However, according to the reports on the effect of chitosan/TPP mass ratio on AE and protein release profiles, it can be concluded that a lower chitosan/TPP ratio produces higher overall release and protein encapsulation (33). It has been explained that a lower viscosity of the gelation medium with lower concentration of chitosan results in a decrease in the liquid phase resistance against dispersion, forming smaller nanoparticles and further promoting protein encapsulation (34, 35).

Also it is noteworthy that the molecular weight of chitosan is an important parameter for determining the optimum chitosan/TPP mass ratio (21, 33). Therefore, according to our results

and the abovementioned reports with regard to optimum encapsulation and overall release, it may be suggested that 2 mg/mL of chitosan and 1 mg/mL TPP concentration are suitable for venom loading. So, in an additional study we have used 2 mg/mL of chitosan and 1 mg/mL TPP for preparation of venom-loaded nanoparticles.

Effect of Venom Concentration on Loading Capacity and Encapsulation Efficiency

In the present study the effect of venom concentration (50, 75, 100, 200, 300, 500, 750 and 1000 µg/mL) has been assessed both on encapsulation efficiency and loading capacity. Our data indicate that elevating the venom concentration from 50 to 75 µg/mL leads to a decrease of encapsulation efficiency, while increasing from 75 to 1000 µg/mL results in an increase in loading efficiency (Figure 3). It seems that in lower concentration of venom there is no significant concentration gradient, and the main factor is the electrostatic reactions between polymer and venom. But, at concentrations higher than 75 µg/mL, the venom concentration plays a major role in loading (21). As shown in Figure 4, the loading capacity of nanoparticles is increased even more than 100% at higher venom concentrations (750 and 1000 µg/mL).

Reports on protein concentration effect on encapsulation are inconclusive and sometimes contradictory. While our findings in this work are in agreement with those of some researchers, they are contrary to Xu and Du (36), who reported that the encapsulation results are reversed on bovine serum albumin (BSA) at pH 6.0 (20, 21, 37, 38). The same trend was also reported by Somnuk J *et al.* (39) for α-lactalbumin, cytochrome C and ribonuclease A. Since protein molecules are large macromolecules with complex 3D structure and the ability to fold and unfold at different solution conditions, their interactions with long cationic chitosan chains and the consequential encapsulation can be complicated, depending on 3D conformation, electrostatic and solution conditions. As a crosslinker and condensing agent, TPP forms additional hydrogen bonds with free amine groups on both protein and chitosan molecules, resulting in more compact protein-chitosan nanoparticles. Additional adsorption of protein molecules on the surface of the formed particles may occur in sequence, leading to additional protein loading on the particles (31). Thus, it is suggested that a venom concentration higher than 500 µg/mL is not suitable for this application, because it may lead to additional adsorption of venom on the surface of formed nanoparticles.

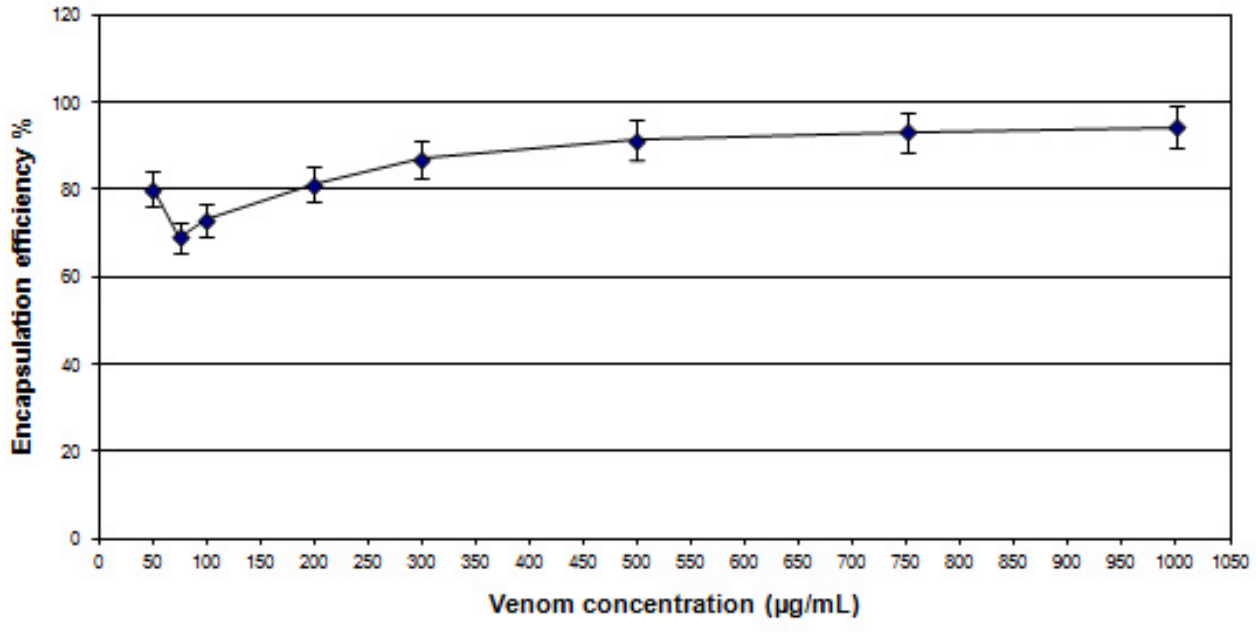

Figure 3. The influence of *M. eupeus* venom initial concentration on encapsulation efficiency (chitosan 2 mg/mL, TPP 1 mg/mL).

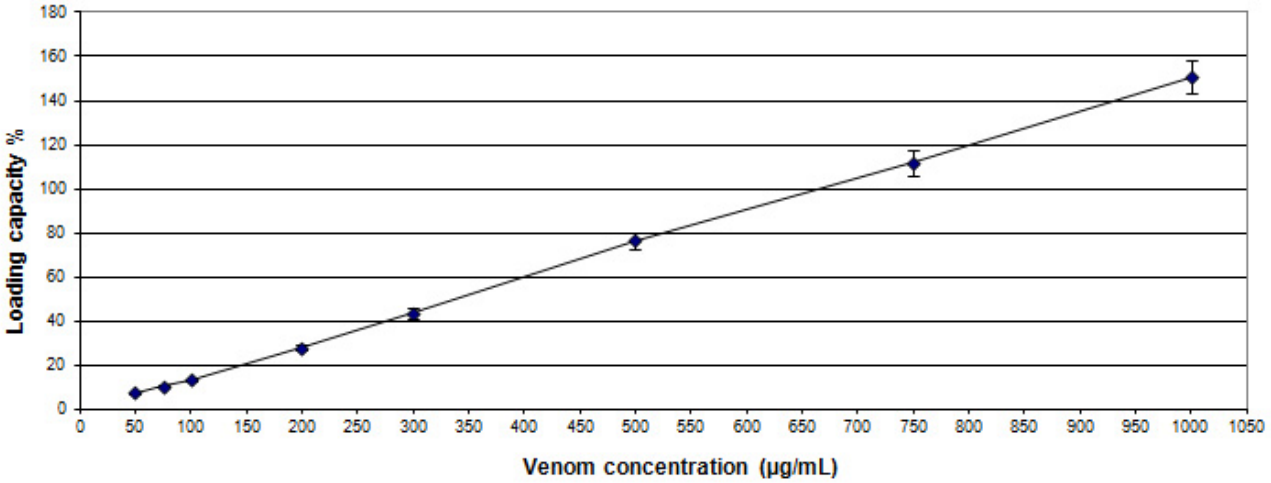

Figure 4. The influence of *M. eupeus* venom initial concentration on loading capacity (chitosan 2 mg/mL, TPP 1 mg/mL).

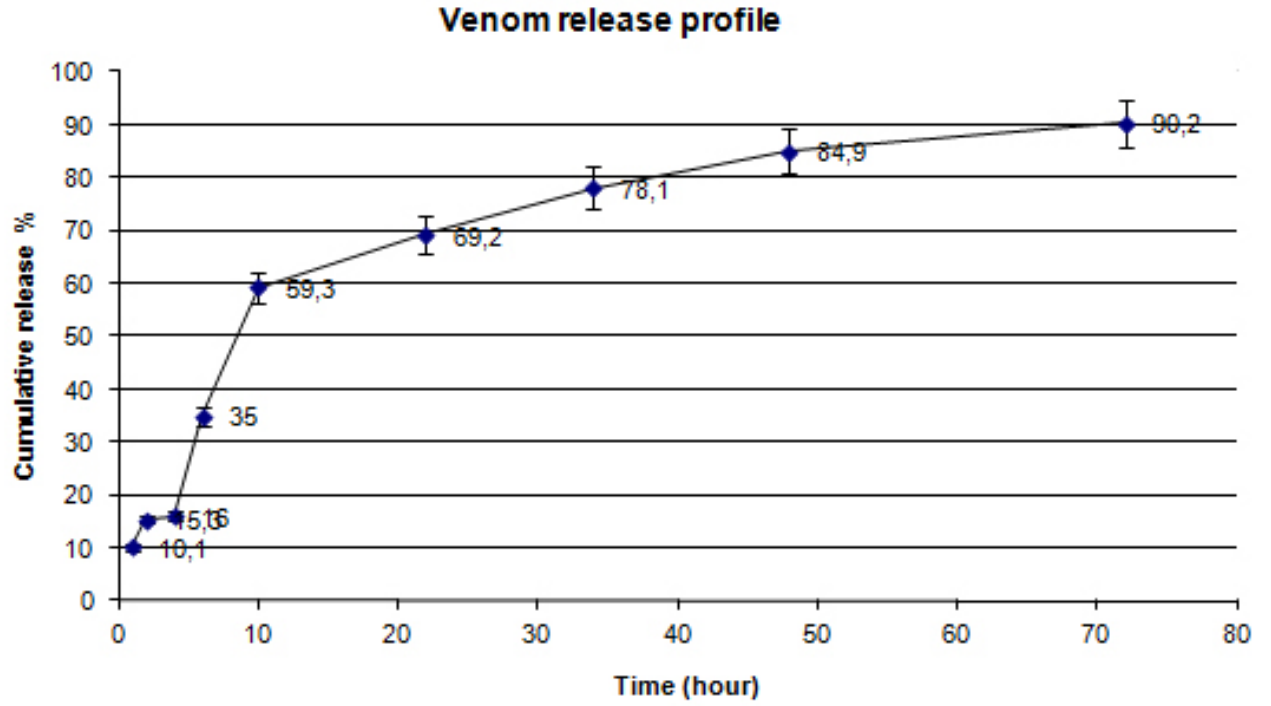

Figure 5. *M. eupeus* venom release profile from venom-loaded chitosan nanoparticles (chitosan 2 mg/mL, TPP 1 mg/mL, initial *M. eupeus* venom 500 µg/mL, medium pH: 7.4).

In vitro Release Study

Our observations showed that about 90% of the loaded venom was released within 72 hours of incubation in PBS. The release profile of venom loaded nanoparticles exhibits an initial burst release of about 60% in the first 10 hours followed by a slow release of 30% for the subsequent 62 hours (Figure 5). The observed burst effect was due to dissociation of protein molecules that were loosely bound to the surface of chitosan nanoparticles (40). In addition, the effect of diffusion of protein molecules dispersing close to the surface of nanoparticles in the first rapid release is undeniable (41). The second part of the release profile is related to the slow release of entrapped protein molecules at an approximately constant rate that arises from the slow degradation of nanoparticles. After 72 hours, the protein degradation rate appears to exceed the release rate (42).

CONCLUSION

In this study, chitosan nanoparticles loaded with *M. eupeus* scorpion venom were prepared based on our recently optimized ionotropic gelation method, which employed TPP as the crosslinker to investigate the physicochemical properties of nanoparticles. We used low molecular weight chitosan in our study. The optimum concentrations obtained for chitosan were 2 mg/mL, venom 500 µg/mL, TPP 1 mg/mL and chitosan/TPP ratio 2:1. Under the abovementioned conditions we have prepared venom-loaded nanoparticles with size range of 300-400 nm, loading capacity of 76.3%, encapsulation efficiency of 91.1% and acceptable PDI. The *in vitro* release study revealed that the release of venom from chitosan nanoparticles could be better sustained than with conventional venom loaded adjuvants. Therefore, the chitosan nanoparticles prepared in our study appear to be an alternative option to traditional adjuvant systems.

SUBMISSION STATUS
Received: June 3, 2011.
Accepted: August 24, 2011.
Abstract published online: August 25, 2011.
Full paper published online: February 28, 2012.

CONFLICTS OF INTEREST
The authors declare no conflicts of interest.

FINANCIAL SOURCE
The Razi Vaccine and Serum Research Institute (Karaj, Iran) provided the financial grants.

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