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A New Antigen Delivery Vehicle Candidate: *Orthochirus iranus* Scorpion Venom Entrapped in Chitosan Nanoparticles

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Authors' contributions

This work was carried out in collaboration between the authors. Author MDN designed the study, wrote the protocol and wrote the first draft of the manuscript and managed the literature searches. The authors read and approved the final manuscript.

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ABSTRACT

Aims: Antivenom has been used successfully for more than a century as the only effective treatment for scorpion stings. *Orthochirus iranus* scorpion venom has high toxicity and preparation of antivenom is necessary for the treatment of its toxic effects. In this study, we prepared *Orthochirus iranus* scorpion venom-loaded nanoparticles as a vehicle to deliver of antigen for the purpose of use for antivenom preparation.
Study Design: Advanced nanotechnology based antigen delivery system study.
Place and Duration of Study: Department of Human Vaccines and Sera, Razi Institute, Karaj, Iran, between May 2011 and August 2014.
Methodology: Nanoparticles were prepared by an ionic gelation method. Factors affecting the loading capacity, efficiency, morphology, surface properties, zeta potential, particle size, particle

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size distribution, and structure of nanoparticles and venom were investigated and optimized.
Results: In optimal conditions, particle size, zeta potential, and particle size distribution of venom-loaded nanoparticles were 83 nm, 36.7 mV, and 0.256, respectively. The nanoparticles showed a smooth surface and spherical shape. The optimal initial concentration of venom was 500 µg/ml with a loading capacity of 80.48% and efficiency of 98.99%. FTIR spectra of nanoparticles and venom-loaded nanoparticles indicated chemical and physical bonds between chitosan, TPP and venom, and evidence of entrapment of venom in the nanoparticle matrix.
Conclusion: We concluded that *Orthochirus iranus* scorpion venom-loaded chitosan nanoparticles could be used for antigen delivery.

Keywords: Orthochirus iranus; scorpion; venom; nanoparticle; chitosan.

1. INTRODUCTION

Nanotechnology is a rapidly expanding field, which encompasses the development of human-made materials in the 5–200 nanometer size range. This dimension vastly exceeds the size of standard organic molecules; but its lower range approaches that of many proteins and biological macromolecules. Nanotechnology has been utilized in medicine for therapeutic drug delivery and development of treatments for a variety of diseases and disorders. Medical therapies have become more tailored to specific diseases and patients in recent years. Nanoparticles (NPs) may be used to achieve therapeutic dosing *via* targeted therapies, establish sustained-release drug profiles, and provide intracellular protection to therapeutic compounds from efflux or degradation [1-3]. Over the past few decades, there has been considerable interest in developing biodegradable NPs as advanced drug delivery systems. Various polymers have been used in drug delivery studies, since they can effectively deliver the drug to a target site and thus increase the therapeutic benefit while minimizing the side effects [4].

Many pharmaceuticals have complications of high toxicity, low water solubility, instability, adverse reactions, etc., which can be avoided if good drug carriers are used [5]. Novel drug delivery systems are required to increase penetration and prevent destruction of vaccines and drugs by hydrolytic and proteolytic enzymes and lysozymse along with increasing stability, target specificity, and solubility in aqueous solutions [6-8].

Biodegradable nanoparticles have a high capacity to transport therapeutic agents including vaccines, medicines, genes, and proteins [9,10]. Biodegradable polymers such as alginate and chitosan have been studied for the delivery of a variety of therapeutic agents including vaccines [11,12]. Chitosan nanoparticles are drug carriers with wide capability and improve stability and solubility, decrease toxicity, and induce the efficacy of drugs by controlled/sustained release of entrapped agents [5]. In the body, biodegradable nanoparticles are metabolized by enzymes to water and carbon dioxide without adverse reactions and have thus become an increasing interest of research [13].

Chitosan is a semi-synthetic polymer and has often better properties than much more expensive synthetic polymers [14-16]. Chitosan is a poly-cationic linear biopolymer with the natural origin formed from β-1,4-linked D-glucosamine and N-acetyl-D-glucosamine [17,18]. It is soluble in dilute acid solutions through the protonation of amine groups [19]. Chitosan has been proven to have the best chelating properties among other natural polymers because of its complex formation of amino groups of chitosan; however, hydroxyl groups can also participate in complex formations [20].

Ionic gelation phenomena can be used easily for the preparation of chitosan nanoparticles using TPP as a crosslinker. This method has a mild status and damaging conditions for proteins, which include high temperature, strongly shaking and destructive organic solvents. Therefore, it could conveniently preserve the activity of biological molecules (such as proteins, nucleic acids, etc.) during nanoparticle fabrication [21]. CS nanoparticles are extensively studied in order to deliver proteins such as tetanus toxoid, diphtheria toxoid, as well as snake and scorpion venom [22-26]. There are numerous polypeptides with various pharmacological and physiological actions in the composition of scorpion venom [27-29].

Scorpions are widely distributed in Iran and its neighboring countries [29]. About 75% of annual mortalities due to scorpion stings in Iran have been reported in the southern half of Iran

(Khuzistan, Sistan and Baluchistan, Hormozgan, and Kerman provinces). However, Khuzistan Province has the highest occurrence rate of death and cases of scorpion stings [30,31]. Iranian scorpion fauna consists of 44 named species from 23 genera in 3 families of Buthidae, Scorpionidae, and Hemiscorpiidae. Khuzistan Province has 19 of these species. *Orthochirus iranus* is one of the healthy important and dangerous scorpion species in Khuzistan Province [32]. In Iran, the total annual incidence of scorpion sting is 140 per 100,000 inhabitants. The patients older than 15 years old represent 55% of the stung victims. Ten to 15% of them are hospitalized and hospital case fatality rate is approximately 1% despite of the use of antivenom [33]. Current polyvalent scorpion antivenom in Iran is prepared against the venoms of *Androctonus crassicauda, Buthotus saulcyi, Buthotus schach, Hemtscorpius lepturus, Mesobuthus eupeus, as well as Odontobuthus dorlae* and could not be used for the treatment of victims by *Orthochirus iranus scorpion.*

The main purpose of the present research was to prepare a new vehicle to deliver antigen in order to improve antivenom manufacturing by progressing the hyper-immunization process of animals. To reach this aim, a novel *Orthochirus iranus* (*OI*) scorpion venom entrapped biocompatible nanoparticles was constructed and its ability as a vehicle for antigen delivery was investigated.

2. MATERIALS AND METHODS

Low molecular weight chitosan (48 kDa) derived from shrimp shells (*Pandalus borealis*), was purchased from Primex Co. (Iceland). The molecular weight of the polymer was determined by viscometry (the degree of deacetylation claimed by the supplier was 95%). Sodium tripolyphosphate (TPP) and coomassie blue G250 and Phosphoric acid (85%), acetic acid and absolute ethanol were purchased from Merck (Germany). *Orthochirus iranus* scorpion 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.

2.1 Preparation of Nanoparticles

Chitosan nanoparticles were synthesized *via* the ionotropic gelation [34–38] of chitosan with TPP anions. Chitosan was dissolved in acetic aqueous solution at 2 mg/mL and pH of 3.9. The concentration of acetic acid in aqueous solution was 1.5 time higher than that of chitosan. The TPP solution (1 mg/mL) was prepared in double-distilled water. Chitosan nanoparticles were spontaneously fabricated with the drop-wise addition of 2 mL of TPP solution to 5 mL of the chitosan solution under magnetic stirring (1100 rpm, 20 min) at room temperature [39–42]. The opalescent suspension was formed under the abovementioned conditions. The nanoparticles were separated by centrifugation at 60,000 ×*g* at 4ºC for 30 min, freeze-dried, and stored at 5± 3ºC. 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 (300, 400, 500, 600 and 700 µg/mL) on nanoparticle's characteristics have been studied. To study one of the abovementioned parameters, the other parameters remained constant.

2.2 Characterization of Prepared Nano-particles

2.2.1 Fourier transform infrared spectro-scopy (FTIR) analysis

The structural features of nanoparticles were estimated by FTIR (Fourier transform infrared) spectroscopy (FTIR- 410® Jasco Colchester, United Kingdom), using KBr pellets.

2.2.2 Surface morphology

The morphology of the particles was examined by Scanning Elecron Microscopy (SEM) (Philips 400, kV 80; Eindhoven, Netherland). The samples were mounted on metal stubs and the stub was then coated with conductive gold with sputter coater attached to the instrument.

2.2.3 Particle size distribution and Zeta potential

The size and surface charge of the nanoparticles were measured with a Malvern zeta sizer (Malvern Instruments, Worcestershire, United Kingdom), based on the dynamic light scattering (DLS) technique. Zeta potential was determined based on the electrophoretic mobility of CS NPs in aqueous suspensions. The particle size distribution is reported as a PDI (polydispersity index). 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 [43,44].

2.2.4 Venom loading efficiency (LE) and loading capacity (LC)

Orthochirus iranus Venom-loaded cross-linked chitosan nanoparticles were centrifuged at 60,000 ×*g* at 4°C for 30 min and the supernatant was removed and subjected to protein (free venom) analysis using the Bradford protein assay at 595 nm [45-47]. LE and LC values were calculated using the following equations (24).

$$LC = (A - B)/C \times 100 \quad (1)$$

$$LE = (A - B)/A \times 100 \quad (2)$$

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

2.2.5 *In vitro* release study of venom-loaded nanoparticles

Venom-loaded chitosan nanoparticles were suspended in separate tubes containing equal volumes of 0.2 mol/L phosphate buffer saline (PBS) solution (pH 7.4) and incubated by shaking at 600 rpm at 37ºC. Appropriate time intervals (1, 2, 4, 6, 8, 24, 30, 48, 72, 96, 120 h) one tube was removed and the sample was centrifuged at 60,000 ×*g* for 30 min. The amount of venom released in the supernatant was then measured.

2.2.6 Kinetic modeling

In order to understand the kinetics and mechanism of venom release, the results of a study on *in vitro* venom release of nanoparticles were fitted with various kinetic equations such as zero order, first order, Higuchi model, and Peppas plot [48-50].

3. RESULTS AND DISCUSSION

3.1 Physicochemical Characterization of Nanoparticles

SEM results showed that the nanoparticles had a nearly spherical shape, a smooth surface, and a size range of approximately 80 nm (Fig. 1). The relevant average diameters, estimated by Zetasizer, of chitosan nanoparticles and venom-entrapped nanoparticles were 69 and 83 nm, resspectively (Fig. 2). The PDI (polydispersity index) values of empty chitosan nanoparticles and venom-entrapped nanoparticles were 0.296 and 0.256, respectively. The outcomes achieved by Zetasizer showed that the venom-entrapped nanoparticles were bigger than the empty nanoparticles, possibly due to the slight rise of the viscosity of loading medium by venom, surface adsorption of venom throughout incubation stage, and high molecular weight and huge mass protein molecules of the venom [51].

Results of the present study indicated the relevant zeta potentials of empty and venom-entrapped nanoparticles of +30 and +36.7 mV (Fig. 3). These outcomes demonstrated that venom entrapping led to the low increase of the zeta potential of the particles. The molecules of chitosan polymer possibly to adopt a dispersed configuration in the solution as a result of electrostatic repulsive enforcement exist among amine groups on the molecular chain. The carboxylic functional groups on the exterior of a huge protein molecule may build hydrogen bonds with amine groups at the specific sites of the chitosan polymer chain, but still retain condensed 3D construction without dispersing in the approximately acidic solution so as to preserve an internal hydrophobic center. Thus, protein molecule binding does not adequately abolish the positive exterior charge of chitosan polymers. This could contribute to be a high ratio of amine groups on the chitosan chain, which remain unoccupied [51,52]. On the other hand, it seems that *Orthochirus iranus* venom proteins possess basic amino acids and relatively acidic solution conditions, which leads to a positive charge of amino groups of these aminoacids. Therefore, the venom entrapment increases the zeta potential of nanoparticles.

FTIR spectra of CS, CS NPs, *Orthochirus iranus* venom-loaded CS NPs and *Orthochirus iranus* venom are shown in Figs 4A–D. In the chitosan spectra (Fig. 4A), the intense and broad peak in the 3000–3600 cm^{-1} region was contributed to hydrogen bonded OH and NH stretching vibration (50). C-O-C stretching vibrations of C-OH appear to be in the area of 1000–1300 cm^{-1}, in the peaks 1078/13 and 1031/85 cm^{-1} [53]. Other vibration peaks, especially those under the 1000 cm^{-1} area, are related to the polysaccharide structure of chitosan.

FTIR spectra of chitosan nanoparticles (Fig. 4B) showed that the O-H stretching region was wider than chitosan polymer spectra, possibly due to the increase in hydrogen bonds in the structure of the nanoparticles. Moreover, a peak at 1541.02 cm^{-1} was related to P=O, which showed the interaction between chitosan and TPP [54,55]. Figs 4D and C shows the FTIR spectra

of the venom and venom-entrapped chitosan nanoparticles, respectively. Comparison between these spectra indicates that the entrapment of venom leads to the shift of some peaks of venom and presence of peaks 2864.65 cm^{-1} and 2923.68 cm^{-1} of venom unchanged in the spectra of venom-loaded nanoparticles. Shifts in some peaks of venom due to encapsulation can be attributed to the chemical interactions between venom proteins and polymer, and the unchanged appearance of some peaks of venom in the spectra of venom-loaded nanoparticles can be attributed to physical interaction and presence of venom in the nanoparticle matrix.

3.2 Effect of Initial Venom Concentration on Loading Efficiency and Loading Capacity

In the present study, the effect of venom concentration (300, 400, 500, 600, and 700 µg/mL) was assessed in terms of both loading efficiency and loading capacity, which can be seen in Table 1. The venom concentration of 500 µg/mL was selected as the optimal concentration and used for release studies.

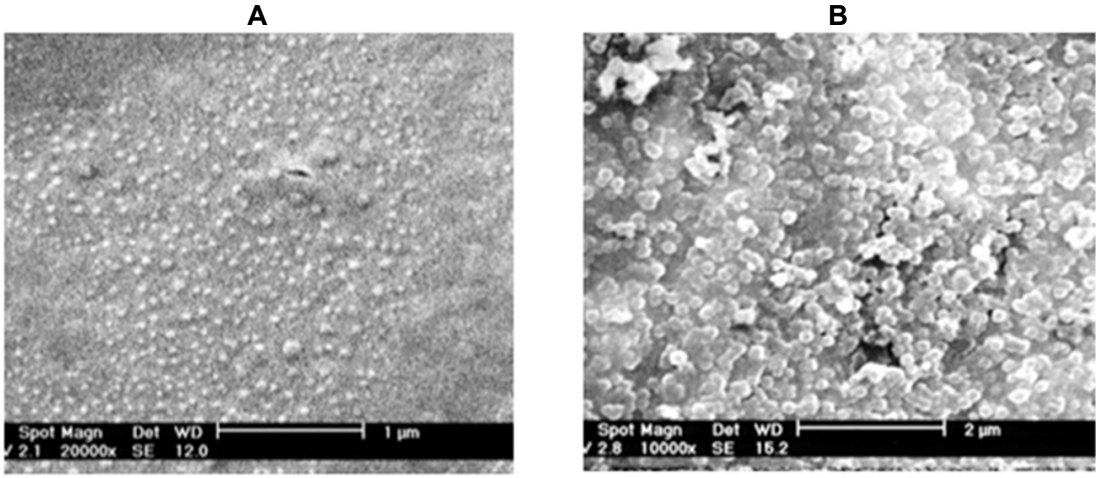

Fig. 1. (A) SEM image of chitosan nanoparticles and (B) *Orthochirus iranus* venom-entrapped chitosan nanoparticles (*Orthochirus iranus* venom 500 µg/mL, TPP 1 mg/mL, chitosan 2 mg/mL)

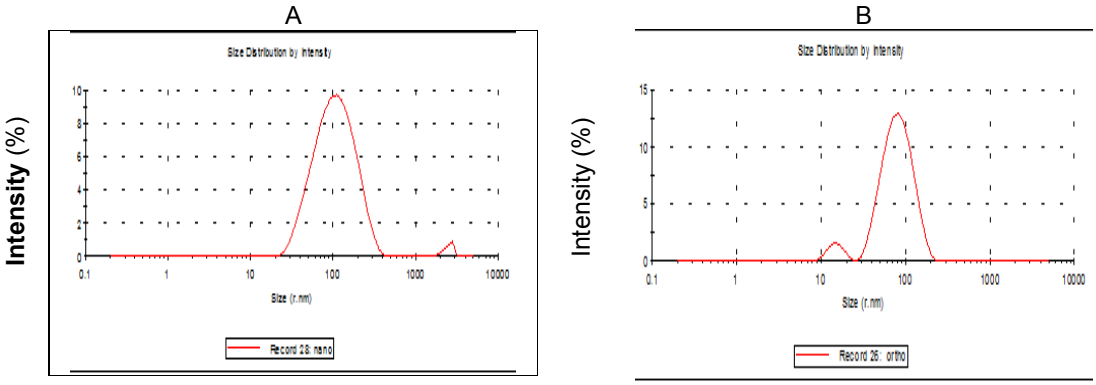

Fig. 2. Size distribution for nanoparticles. (A) chitosan nanoparticles and (B) *Orthochirus iranus* venom-entrapped chitosan nanoparticles (*Orthochirus iranus* venom 500 µg/mL, TPP 1 mg/mL, chitosan 2 mg/mL)

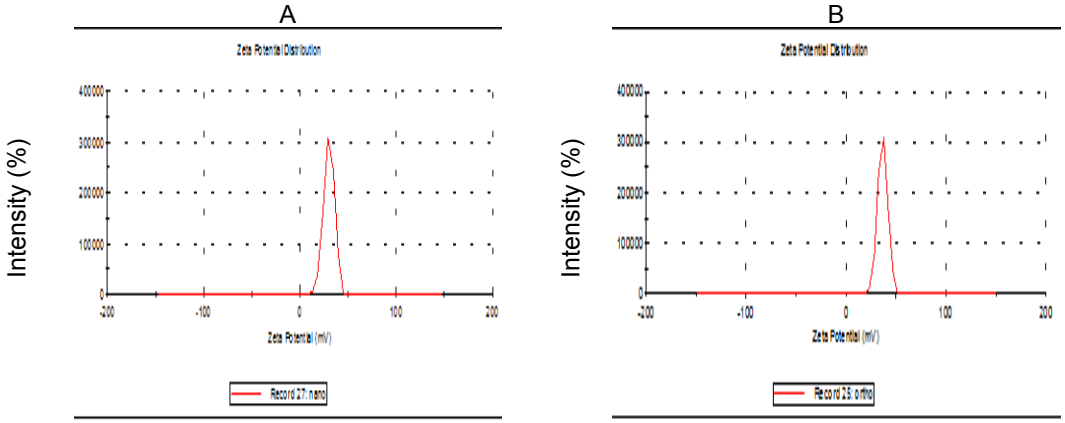

Fig. 3. Z potential of nanoparticles.(A) chitosan nanoparticles and (B) *Orthochirus iranus* venom-entrapped chitosan nanoparticles (*Orthochirus iranus* venom 500 µg/mL, TPP 1 mg/mL, chitosan 2 mg/mL)

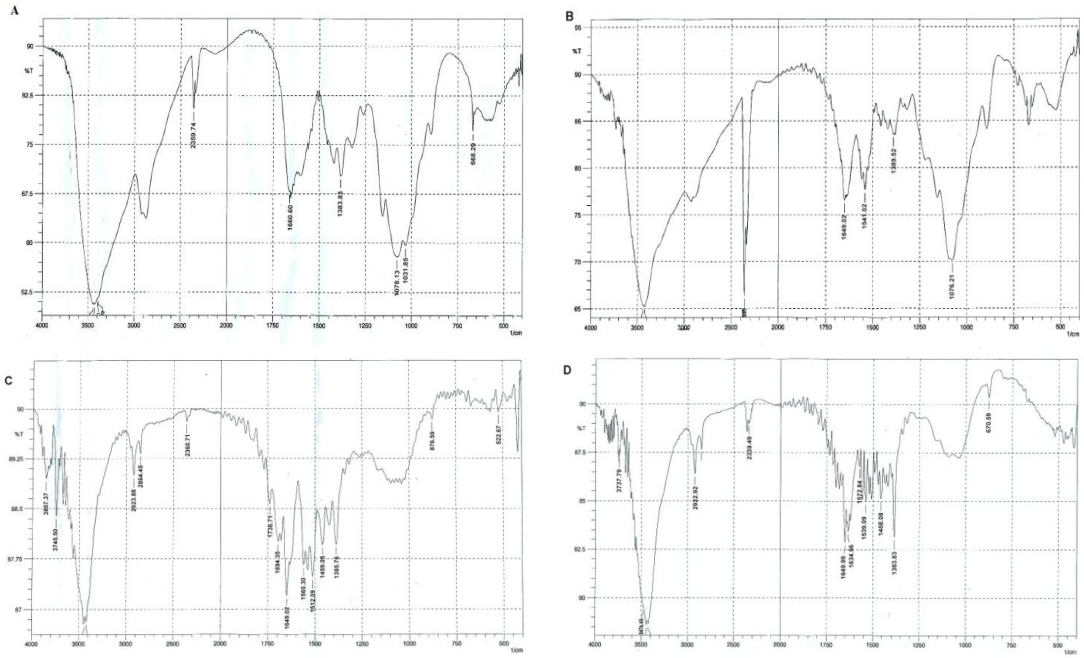

Fig. 4. FTIR spectrum of chitosan (A), empty chitosan nanoparticles (B), *Orthochirus iranus* venom-loaded chitosan nanoparticles (C) and crude *Orthochirus iranus* venom (D)

Table 1. The effects of different initial concentrations of *Orthochirus iranus* scorpion venom on LE and LC of nanoparticles (n=3)

Venom initial concentration (µg/mL)	LE (%)	LC (%)
300	76.65±3.81	66.30±3.45
400	83.00±2.93	71.00±2.70
500	99.98±3.35	80.44±3.15
600	99.80±2.34	100.12±3.00
700	99.12±2.40	133.40±2.55

TPP creates excessive hydrogen bonds with free amine groups on both chitosan and protein molecules, as a condensing and cross-linker agent, and leads to denser chitosan-protein nanoparticles. Further protein molecule adsorption on the surface of the constructed nanoparticles may happen in sequence, resulting in extra protein entrapping on the nanoparticles [51,52]. Therefore, it seems that initial concentration of venom more than 500 µg/mL is not appropriate for loading because it can result in extra venom adsorption on the surface of the particles.

3.3 *In vitro* Release Study

Studies of venom release from venom-entrapped nanoparticles were performed employing the solution of PBS (pH 7.4) and protein detection was done by the Bradford method. The present results showed that approximately 70% of the entrapped venom was released during 96 h of incubation in phosphate buffer saline. The pattern of release from venom-entrapped nanoparticles showed an starting surge release of about 25% in the first 24 h subsequent to the gradual release of 45% for the following 72 h (Fig. 5). The noticed burst release was the result of dissolving protein molecules that were closely bound to the exterior of venom-entrapped chitosan nanoparticles [56]. Moreover, the result of the dissociation of protein molecules disseminating loosely to the nanoparticle surfaces in the initial burst release was formerly reported in [52]. The second segment of the release pattern was related to the gradual release of loaded protein molecules at an about steady rate that originated from the gradual degeneration of nanoparticles. The protein destruction rate appeared to overtake the release rate after 96 h [57].

3.4 Kinetic Studies of Venom Release

In order to describe the release kinetics, the corresponding *in vitro* release data of *Orthochirus iranus* venom were fitted in various kinetic release models including zero order, first order, and Higuchi (Figs. 6–8, respectively). As indicated in Figs. 6 and 8, higher R^2 (coefficient of correlation) values for venom release from venom-loaded nanoparticles followed zero order (R^2= 0.8979, Fig. 6) and Higuchi model (R^2= 0.9664, Fig. 8). The high correlation coefficient value (R^2) of venom release from venom-loaded nanoparticles in Higuchi model confirmed that the main release mechanism was swelling and diffusion controlled.

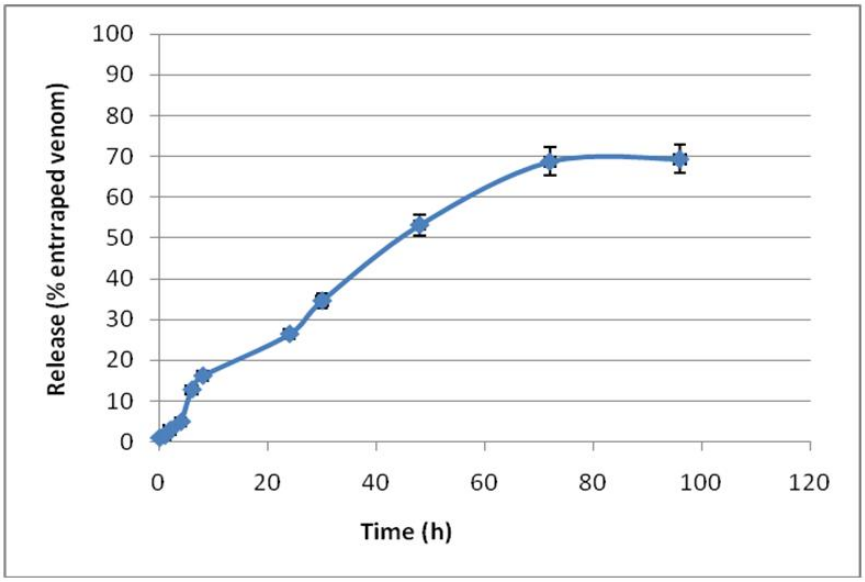

Fig. 5. *In vitro* release pattern of *Orthochirus iranus* venom from venom-entrapped chitosan nanoparticles (*Orthochirus iranus* venom 500 µg/mL, TPP 1 mg/mL, Chitosan 2 mg/mL), n=3

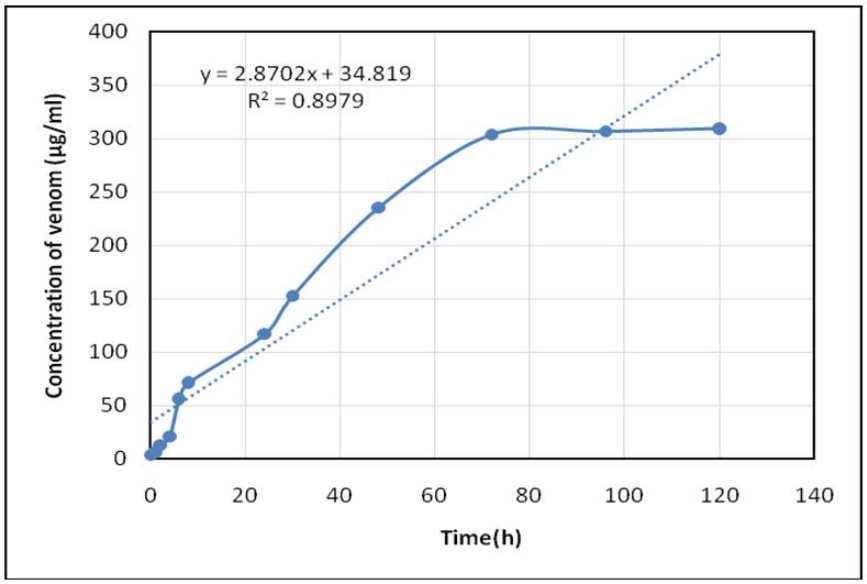

Fig. 6. Zero order release kinetic curve of *Orthochirus iranus* venom from venom-entrapped nanoparticles of chitosan (initial *Orthochirus iranus* venom concentration 500 µg/mL, chitosan 2 mg/mL, TPP 1 mg/mL, release medium pH: 7.4)

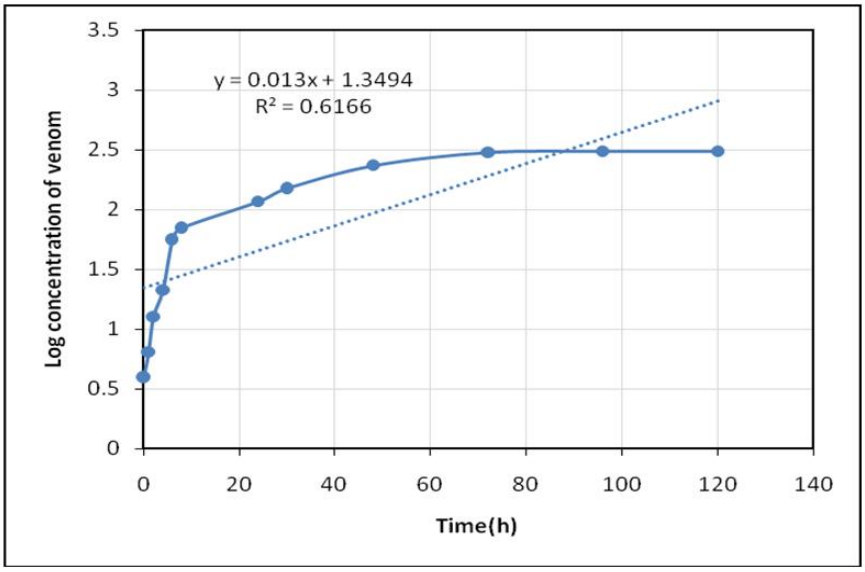

Fig. 7. First order release kinetic curve of *Orthochirus iranus* venom from venom-entrapped nanoparticles of chitosan (initial *Orthochirus iranus* venom concentration 500 µg/mL, chitosan 2 mg/mL, TPP 1 mg/mL, release medium pH: 7.4)

As shown in Fig. 5, the initial burst release of venom from venom-loaded nanoparticles was very little and the main portion of entrapped venom released slowly. This point is an important advantage of this system in the antivenom industry, because the sustained release of venom leads to good stimulation of the immune system. Moreover, small burst release decreases adverse effects and problems due to venom in the hyperimmunization process in animals.

The study for antivenom production by *T. serrulatus* scorpion venom-entrapped CN NPs compared to aluminum hydroxide, the traditional adjuvant, revealed that chitosan nanoparticles are a promising and safe system for

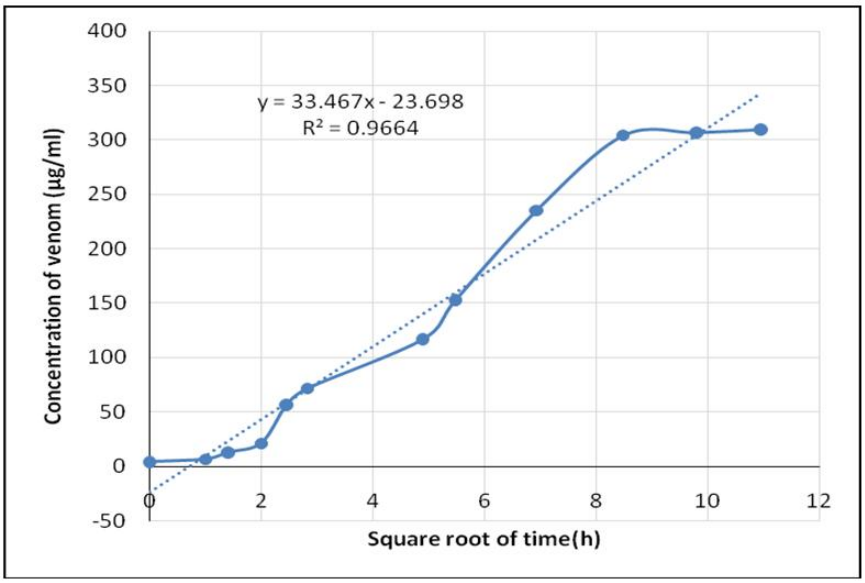

Fig. 8. Higuchi's model release kinetic curve of *Orthochirus iranus* venom from venom-entrapped nanoparticles of chitosan (initial *Orthochirus iranus* venom concentration 500 µg/mL, chitosan 2 mg/mL, TPP 1 mg/mL, release medium pH: 7.4)

peptide/ protein delivery [25]. Currently used traditional adjuvants (water in oil emulsion) in the manufacturing process of antivenom for the immunization of animals create some problems; additionally, they have only weak immunostimulating ability. CS NPs have different desirable properties from the conventional adjuvants and antigen delivery systems: excellent versatility in preparing derivatives with different loading ingredients; cheapness; easy access to polymers; high loading capacity; long-term release (a crucial advantage for immunization process); improved stability of entrapped agents; flexible modes of administration; reduction in the number of required injections, fewer side effects (such as pain and ulcer), delivering greater amount of intact antigens into the body, allowing targeted delivery of antigens to immune cells, and higher immunostimulating capacity [24,41,58-60].

4. CONCLUSION

In the present study, *Orthochirus iranus* venom-entrapped nanoparticles of chitosan were constructed with desirable physicochemical and biological properties. The *in vitro* release investigation showed that the venom release from venom-entrapped nanoparticles had a slight burst effect, high correlation coefficient value (R^2) of venom release in Higuchi model, and a good sustained release profile. It can be concluded that the *Orthochirus iranus* venom-entrapped nanoparticles, if used as an antigen delivery system, could result in good immunogenicity and few side effects. Therefore, the venom-loaded chitosan nanoparticles developed in this research may be used as an advanced adjuvant and antigen delivery system for antivenom purposes.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

ACKNOWLEDGEMENTS

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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