Nanomed Res J 4(4):220-227, Autumn 2019

RESEARCH ARTICLE

Characterizing and controlling the loading of *Vipera albicornuta* venom in chitosan nanoparticles as an adjuvant and vaccine delivery system

Mohammad Hassan Shambayati [1], Mohsen Mehrabi [2], Nasser Mohammadpour Dounighi [3,*], Ali Ramazani [1], Abbas Zare Mirakabadi [3], Ebrahim Ahmadi [1]

[1] Department of Chemistry, Zanjan University, Zanjan, Iran

[2] Department of Medical Nanotechnology, School of Medicine, Shahroud University of Medical Sciences, Shahroud, Iran

[3] Department of Human Vaccines and Serum, Razi Vaccine and Serum Research Institute, Agricultural Research,Education and Extension Organization Karaj, Iran

ARTICLE INFO

Article History:
Received 04 September 2019
Accepted 07 November 2019
Published 15 November 2019

Keywords:
Chitosan
Vipera albicornuta venom
Nanoparticles
Antigen delivery

ABSTRACT

The purpose of this research is designing and preparing the chitosan nanoparticles (CS-NPs) loading with snake (*Vipera albicornuta)* venom as well as evaluating the influence of different factors on the encapsulation efficiency (EE) and loading capacity (LC) of venom. The morphologies and characteristics of CS-NPs were specified respectively through scanning electron microscopy (SEM) and Fourier transform infrared spectroscopy (FT-IR). CS-NPs were fabricated based on the ionic gelation method of tripolyphosphate (TPP) and chitosan (CS). The results showed a smooth surface, spherical shape by SEM. The particle size of loaded nanoparticle was 187 nm and zeta potential was found to be 46.7 mV. Optimum concentration obtained 400 µg/ml for loading of venom, which leads to LC 86% and EE 91%. Based on the results of the study, it can be concluded that the *vipera albicornuta* venom loaded CS-NPs may be used as a new antigen delivery system.

How to cite this article
Shambayati MH. Mehrabi M, Mohammadpour Dounighi N, Ramazani A, Zare Mirakabadi A, Ahmadi A. Characterizing and controlling the loading of Vipera albicornuta venom in chitosan nanoparticles as an adjuvant and vaccine delivery system. Nanomed Res J, 2019; 4(4): 220-227. DOI: 10.22034/nmrj.2019.04.003

INTRODUCTION

Chitosan (CS) has shown high potential for a wide range of pharmaceutical purposes [1, 2]. Many studies have been conducted to evaluate the use of CS in the design of sustained release dosage forms, such as matrix tablets, and have shown that CS generally provides magnificent sustained release properties *in vitro*, especially at low pHs where a gel matrix is developed [3, 4]. A large number of studies have been recently performed on polymer nanoparticles as carriers for delivery of drugs and antigens [5, 6]. CS-NPs were investigated as an adjuvant and vaccine delivery system[7-9]. The absorption enhancing effect of CS-NPs has been proven to improve the mucosal immune response. The mechanism of CS-NP to improve the delivery of antigens across mucosal membranes can be explained using the same theory discussed earlier in the section about peroral administration [10, 11]. Researchers successfully developed CS and CS-NPs vaccines containing antigens of influenza, pertussis, and diphtheria for nasal delivery. They revealed that the vaccines produced a significant amount of antibody in mice, which included serum and secretory Immunoglobulin A (IgA) [12-15]. Besides the potential carrier for mucosal delivery vaccines, it has been reported that CS-NPs could act as an adjuvant for systemic vaccine delivery, such as making the macrophages and poly morphonuclear cells further proliferate and active. Macrophages are activated once the CS-NPs have been absorbed [16-18]. Today, to produce the antivenom, usually

* Corresponding Author Email: *nasser_mohammadpour@yahoo.com*

antigen are delivered to the horse mostly via intramuscular route [19]. These delivery routes have several weaknesses: high initial concentrations of the antigen that cause side effects. Another reason is related to requirement of multiple injections, because only a very small percentage of the injected antigen reaches the affected area to induce enough immune system. It is therefore important to reduce the number of repeated venom administrations required for immunization over a long period of time and improvement of the production yield [7, 20, 21].

This study was thus conducted to examine the influence of a number of factors on the encapsulation of *Vipera albicornuta* and evaluate physicochemical properties of CS-NPs supposed to be used as a new antigen delivery system, using FTIR, DLS, and SEM.

MATERIALS AND METHODS

Materials

The medium molecular weight (MMW) CS derived from shrimp shells (Pandalus borealis) was purchased from Primex Co (Iceland). The degree of deacetylation was a minimum of 95%. Sodium tripolyphosphate (TPP) and coomassie blue G250 were supplied from Sigma (USA). Phosphoric acid (85%), acetic acid, and absolute ethanol were purchased from Merck (Darmstadt, Germany). *Vipera albicornuta* venom, prepared as a freeze-dried powder, was provided from Razi Vaccine and Serum Research Institute (Karaj, Iran). Other materials were of pharmaceutical and analytical grades and were used as they were received.

Nanoparticle preparation and venom-loaded

The CS-NPs were prepared using ionic gelation technique of CS with TPP anions [22, 23]. Different concentrations of CS (1.5, 2.0, 2.5, 3.0 mg/mL) were dissolved in acetic acid aqueous solution. Solutions of TPP with different concentrations were prepared afterwards. Eventually, 5 mL of the CS solution was added to 2 ml of the TPP solution dropwise under constant magnetic stirring (1100 rpm, 1 hour) at room temperature. An opalescent suspension formed spontaneously under the above-mentioned conditions. The nanoparticles were separated through centrifuging the suspension at 11,200 g, 14°C for 30 minutes. The freeze-dried nanoparticles were then weighed and stored at 4°C–8°C. In this study, the influence of different parameters, including venom concentrations (20, 30, 40, 50, 100, 200, 300, 400, 500, 750, and 1000 µg/mL), TPP concentrations (0.75, 1 and 1.25 mg/mL) and CS concentrations (1.5, 2, 2.5, and 3 mg/mL) on EE and LC was assessed. Once one of the above-mentioned parameters was under evaluation, the other parameters were kept constant.

Characterization of morphology and structure of nanoparticles

The characterization of morphology and particle size was carried out using a scanning electron microscope (SEM) (INCA-500, United Kingdom) as an accelerating voltage of 15.0 kv after sputtering with gold. The CS-NPs separated from suspension were dried with a freeze dryer, and the FTIR was performed using KBr pellets on Bomem Spectrum. The dynamic light scattering technique was performed to measure the particles' size, size distribution [polydispersity index (PDI)], and zeta potential, using Zetasizer (Malvern Instruments, UK) [24, 25].

Venom loading percentage

The venom-loaded nanoparticles were removed from the aqueous suspension through centrifugation of the suspension at 11,200 g and 14°C for 30 minutes. The supernatant was removed, and the protein content (free venom) of the supernatant was determined through the Bradford protein assay spectrophotometric method at 585 nm. Equations 1 and 2 were used to respectively calculate the venom LC of nanoparticles and the venom EE of the process. Equation as follows [26, 27]:

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

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

A: Total amount of venom; B: Amount of free venom; C: Nanoparticle weight.

Studies on in vitro release of venom from CS-NPs

A certain amount of venom-loaded CS-NPs was suspended in separate test tubes containing equal volumes of 0.2 mol/L PBS solution (pH 7.4) and incubated at 37°C and 600 rpm. At appropriate time intervals (1, 2, 4, 6, 10, 22, 34, 48, 72 hours), the content of one tube was removed and centrifuged at 19,000 g and 10°C for 35 minutes. The Bradford assay was performed to measure the amount of venom released [28].

RESULTS AND DISCUSSIONS

Characterization of nanoparticles in terms of physiochemical properties

Fig. 1 shows morphological characteristics of nanoparticles. As shown in Fig. 1A as well as Fig. 1B, the shapes of particles are almost similar to each other with smooth semi-spherical or nearly oval appearance. According to DLS results, the mean size of the particles was 155 and 187 nm for CS and loaded CS-NPs, respectively. The mean size of empty and loaded nanoparticles was found to be bigger than that determined by SEM. In comparison with the SEM, the DLS technique measures the hydrodynamic diameter of the particles, including hydration layer and polymer shells, and generally shows a larger particle size [29]. The aggregates seen in SEM images could be attributed to the drying process during sample preparation [30]. The PDI value of CS was approximately the same as venom loaded nanoparticles (0.500 and 0.577), it means that no significant change was observed in PDI (Table 1).

The stability of venom loaded CS-NPs is related to Zeta potential [31]. The particles' surface charge was somewhat positive, with CS zeta potentials of 46.7 mV and venom loaded nanoparticles of 33.2 mV. The result of this study indicated that venome loading can cause minor reduction in Zeta potential. The venome involves with long chains of CS molcules, and it is not uniform. Due to electrostatic repulsive forces existing between amine groups and the molecular chain, CS molecules probably adopt a diffuse conformation in the solution. On certain sites along the CS chain, hydrogen-amine group bonds might form by carboxyl groups on a large proten molecule's surface. The CS chain, however, still maintains its own compact three-dimensional structure without diffusing in the relatively acidic solution in order to keep its hydrophobic core [28]. The positive surface charge of CS molecules is thus not suppressed adequately by the protein molecule attachment. There are still possibly a large number of unoccupied amine groups on the CS chain [31].

The potential of the ionic gelation process for formation of venom-loaded CS-NPs was evaluated

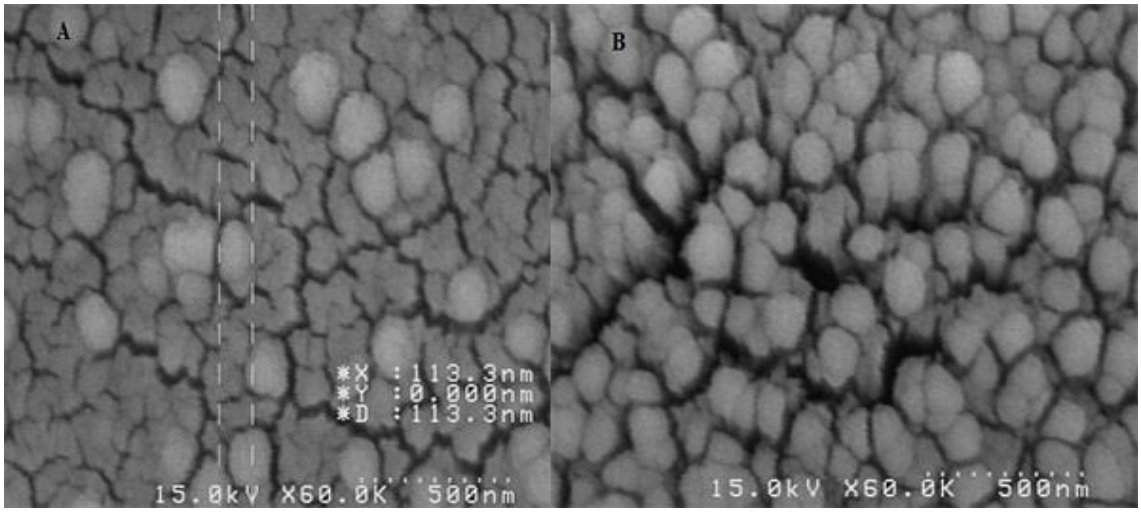

Fig. 1. (A) SEM image of CS-TPP nanoparticles and (B) Vipera albicornuta venom-loaded CS-TPP nanoparticles (CS: 2.5 mg/mL; Vipera albicornuta venom 400 µg/mL)

Table 1. Particle size, PDI and zeta potential of CS-NPs and venom-loaded by DLS.

CS concentration (mg/mL)	Particle size (nm)		Polydispersity index (PDI)		Zeta potential (mV)	
	Without venom loading	Venom loaded nanoparticles	Without Venom loading	Venom loaded nanoparticles	Without Venom loading	Venom loaded nanoparticles
2	1340	NM	1	NM	56.2	NM
2.5	155	187	0.50	0.582	46.7	33.2
3	2034	NM	1	NM	51.2	NM

Particle preparation conditions: venom concentration 400 µg/mL, with 1 mg/mL TPP, T: 25 ± 2°C. NM: not measurement (the physicochemical characteristics of nanoparticles prepared with CS concentrations 2 and 3 mg/mL is not suitable for further study).

through employing FTIR to detect venom-CS interactions. Fig. 2 shows the FTIR spectra of CS-NPs, venom loaded CS-NPs, and venom. In CS-TPP nanoparticles, the wide peak in the 3000 cm^{-1}–3500 cm^{-1} range, related to the combined peaks of O-H stretching and intermolecular hydrogen bonding, led to decrease bending vibration of amine group. Similar to the primary amines, the N-H stretching is overlapped in the same area. The bond at 3420 cm^{-1} is related to to NH_2 and OH group stretching vibration in CS. The peak around 1075 cm^{-1} is related to the symmetric stretch of C-O-C that usually appears between 1000 to 1300 cm^{-1} and 1320 cm^{-1} representing the C-N stretching vibration. Other peaks under 1000 cm^{-1} are related to the CS polysaccharide structure. Furthermore, the crosslinked CS indicates a P=O peak at 1157 cm^{-1} [12]. These results might arise from the link between phosphoric and ammonium ions. It was therefore supposed that the tripolyphosphoric groups of TPP were linked with the ammonium group of CS, and the inter- and intramolecular actions are improved in CS-NPs. The spectrum of *Vipera albicornuta* venom-loaded CS-TPP nanoparticles and venom were compared, it showed the same peak at 1455 cm^{-1} and 1541 cm^{-1}, so we conclude that the structure of venom remained intact in loading duration [27, 28].

Effects of CS concentrationss

A particle size analyzer was applied to evaluate the effect of different CS concentrations (2, 2.5, & 3 mg/mL) on particle size. The results showed that an increase in the CS concentration from 2 mg/ml to 3 mg/mL while the TPP concentration (1 mg/mL) was constant, led to an increase in the size of nanoparticles (Table 1). The nanoparticles with CS concentrations of 2 mg/ml and 3 mg/mL did not show a PDI value within the acceptable range due to the formation of large-diameter aggregates. The PDI value of nanoparticles was desirable at the CS concentration of 2.5 mg/mL. The results also revealed the effect of CS/TPP mass ratio on EE and protein release profiles. It can be therefore concluded that lower CS/TPP ratios lead to higher encapsulation of proteins [32]. Resistance of liquid phase decreases against dispersion with a lower concentration of CS leading to formation of smaller size of nanoparticles and increase venom encapsulation [33, 34]. The molecular weight of CS is generally a significant factor for specifiying the optimum CS/TPP mass ratio [32, 35]. Considering the results of this study and results of previous studies about the optimum encapsulation and overall release, it may be argued that the venom loading is well supported using the CS concentration of 2.5 mg/mL and TPP concentration of 1 mg/mL.

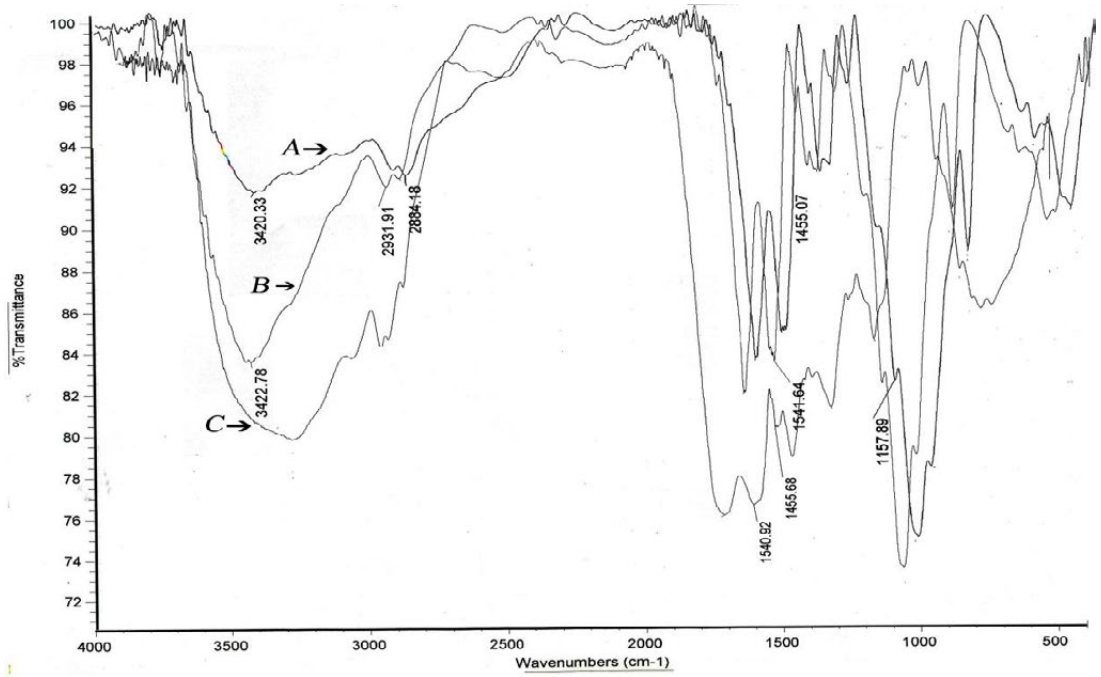

Fig. 2. FT-IR spectra of CS (A), CS-TPP nanoparticles (B) and Vipera albicornuta venom (C).

Effects of venom concentrations on LC and EE

The effect of venom concentrations (20, 50, 100, 200, 300, 400, 500, 750 and 1000 µg/mL) on EE and LC was examined in this study. The results indicated that an increase in the venom concentration from 20 µg/mL to 750 µg/mL led to an increase in EE though it remained unchanged atvenom concentrations higher than 750 µg/mL, (Fig. 3). The results also showed that the concentrations higher than 50 µg/mL of venom highly contributed to the venom loading [28]. As shown in Fig. 4, the nanoparticles' loading capacity increased even more than 100% at higher venom concentrations (400 µg/mL to 1000 µg/mL). Previous reports on the effect of protein concentration on encapsulation are incomprehensive and sometimes contradictory. However, this study conforms to those of some researchers. The results of this study do not agree with those obtained by Xu and Du [36], who reported reverse results about encapsulation on bovine serum albumin (BSA) at pH 6.0. Jarudilokkul S et al. also reported the same result for α-lactalbumin, cytochrome C, and ribonuclease A [37]. As a cross linking agent, TPP may react with free amine groups on both protein and CS molecules, developing more compact protein CS-NPs. The amount and size of protein on the surface of the nanoparticles increase by the excessive adsorption of protein on the surface of the nanoparticles [37]. In this respect, the suggestion is that venom concentrations higher than 400 µg/mL are not suitable for this application, because they may lead to additional adsorption of venom on the surface of nanoparticles, which in turn results in a decrease in zeta potential and aggregation of particles [28].

The antivenom product is derived from equine hyperimmune serum and capable of neutralizing the poison of the six greatest common snakes in Iran, including 5 viper species (*Echis*

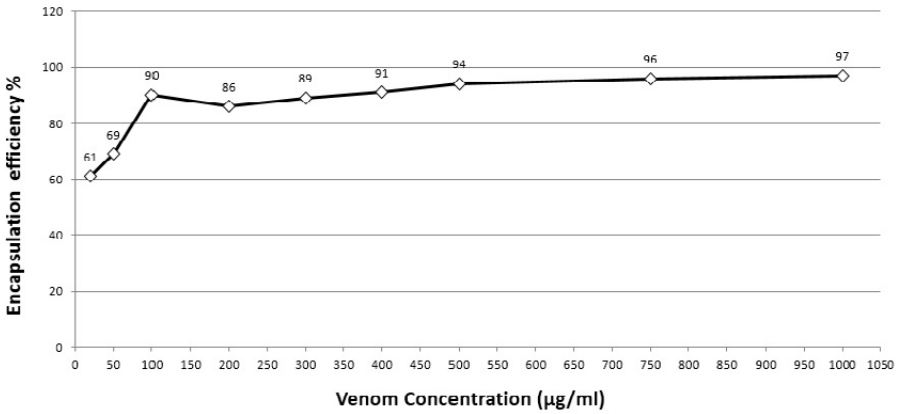

Fig. 3. The influence of Vipera albicornuta venom initial concentration on EE (CS: 2.5 mg/mL, TPP: 1 mg/mL).

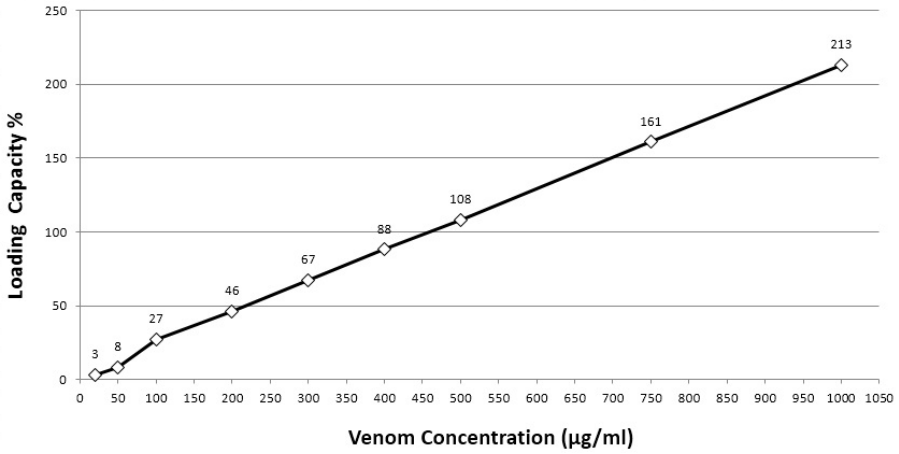

Fig. 4. The influence of Vipera albicornuta venom initial concentration on LC (CS: 2.5 mg/mL, TPP: 1 mg/mL).

Table 2. Effect of MW-CS on percentage of LC and EE for different venoms.

Type of venom at optimum Concentration (µg/mL)	Concentration of TPP (mg/mL)	Concentration of CS (mg/mL)	Loading Capacity (%) (LC)	Encapsulation efficiency (%) (EE)	MW of CS	References
Naja naja oxiana (500µg/mL)	1	2	53.4	86	Low	[39]
Mesobuthus eupeus (500 µg/mL)	1	2	76.3	91.1	Low	[28]
Hemiscorpius lepturus (500 µg/mL)	1	2	89.4	94.7	Low	[40]
Vipera albicornuta (400 µg/mL)	1	2.5	86	94	Medium	

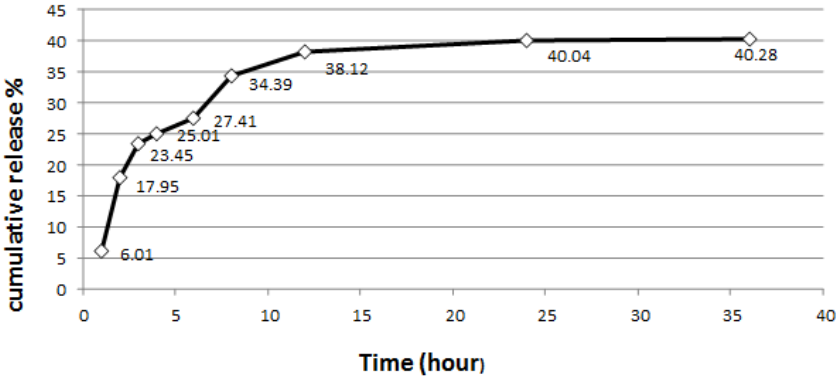

Fig. 5. Vipera albicornuta venom release profile from venom-loaded CS-NPs (CS: 2.5 mg/mL, TPP: 1 mg/mL, initial Vipera albicornuta venom: 400 µg/mL and pH: 7.4).

carinatus sochureki, Vipera lebetina obtusa, Vipera albicornuta, Agkistrodon halys, and *Pseudocerastes persicus*) and an elapid species (*Naja naja oxiana*) [38]. This product has been professionally used to treat snakebites.

Our group previously reported the CS-NPs loaded with various antigens such as *Naja-Naja Oxiana* [39], *Mesobuthus eupeus [28]*, *Hemiscorpius lepturus* [40], *Echis Carinatus* [27] and *Tetanus toxoid* (under publication). For *Naja-Naja Oxiana,* the optimum LC and EE of venom at a concentration of 400 µg/mL and TPP concentration of 1 mg/mL were obtained for LMW of CS at a concentration of 2 mg/mL. In respect of the *Mesobuthus eupeus*' optimum encapsulation and loading capacity, it may be suggested that 2 mg/mL of CS and 1 mg/mL of TPP are suitable concentrations for 400 µg/mL venom loading (LMW CS). Moreover, optimum EE and LC were observed when the CS concentration (with low-MW CS) and *Hemiscorpius lepturus* venom were 2 mg/mL and 400 µg/mL, respectively. But in our study we used CS with MMW. In comparison, report of Mohammadpour, et al for Naja-Naja Oxiana, Mesobuthus eupeus and Hemiscorpius lepturus venom, with LMW of CS loading capacity can be increased about Vipera albicornuta venom with MMW of CS, however we could reduce concentration of venom (Table 2). Also Table 2 shows greater EE for MMW-CS-NPs than that for LMW-CS. This is probably caused by the longer chains of MMW CS, which can generate higher amount of venom when being gelated with TPP.

In vitro release

The result of this study revealed that about 40% of the loaded venom was released during the first 36 hours of incubation in PBS. The release of venom from the NPs showed an initial burst release within initial hours of incubation followed by slow release for 40 hours (Fig. 5). The observed burst was caused by the breakdown of protein molecules that were loosely bound to the surface of CS-NPs and released from the surface of nanoparticles. [11, 41]. Regarding the second part of the release profile, the slow release of entrapped protein molecules at an approximately constant rate arouse from the slow decomposition of nanoparticles. After 36 hours, the protein degradation rate preceded the release rate [42].

CONCLUSION

In this study, CS-NPs loaded with *Vipera albicornuta* venom were prepared through recently recently optimized ionotropic gelation method, using TPP as the crosslinker to examine the physicochemical properties of nanoparticles. The MMW chitosan was used in this study. The optimum concentrations of CS, venom, and TPP were respectively 2.5 mg/mL, 400 µg/mL, and 1 mg/mL; the optimum CS/TPP ratio was 5:2. Venom-loaded nanoparticles with size of 187 nm, LC of 86%, EE of 94% and acceptable PDI were prepared under the above-mentioned conditions. Moreover, it was found that the controlled release properties may be affected by different factors, such as CS and venom concentration. Therefore, the CS-NPs prepared in this study appears to be an alternative for conventional delivery systems.

ACKNOWLEDGEMENTS

The researchers hereby appreciate the laboratory staff and library staff in Razi Vaccine and Serum Research Institute (RVSRI) for their assistance.

CONFLICT OF INTEREST

The researchers report no conflicts of interest and are responsible for the content and writing of the article.

REFERENCES

1. Ilium, L., *Chitosan and its use as a pharmaceutical excipient.* Pharmaceutical research, 1998. **15**(9): p. 1326-1331.
2. Li, C. and J.-B. Li, *Preparation of chitosan-ferulic acid conjugate: Structure characterization and in the application of pharmaceuticals.* International Journal of Biological Macromolecules, 2017.
3. Kawashima Y, Handa T, Kasai A, Takenaka H, Lin SY. The effects of thickness and hardness of the coating film on the drug release rate of theophylline granules coated with chitosan-sodium tripolyphosphate complex. CHEMICAL & PHARMACEUTICAL BULLETIN. 1985;33(6):2469-74.
4. Patel BK, Parikh RH, Aboti PS. Development of Oral Sustained Release Rifampicin Loaded Chitosan Nanoparticles by Design of Experiment. Journal of Drug Delivery. 2013;2013:1-10.
5. Gref R, Minamitake Y, Peracchia M, Trubetskoy V, Torchilin V, Langer R. Biodegradable long-circulating polymeric nanospheres. Science. 1994;263(5153):1600-3.
6. Ogunjimi AT, Melo SMG, Vargas-Rechia CG, Emery FS, Lopez RFV. Hydrophilic polymeric nanoparticles prepared from Delonix galactomannan with low cytotoxicity for ocular drug delivery. Carbohydrate Polymers. 2017;157:1065-75.
7. Mirzaei, F., M.R. Avadi, and M. Rezayat, *A New Approach to Antivenom Preparation Using Chitosan Nanoparticles Containing Echis Carinatus Venom as A Novel Antigen Delivery System.* Iranian Journal of Pharmaceutical Research, 2017. **16**(3): p. 858-867.
8. Izaguirre-Hernández IY, Mellado-Sánchez G, Mondragón-Vásquez K, Thomas-Dupont P, Sánchez-Vargas LA, Hernández-Flores KG, et al. Non-Conjugated Chitosan-Based Nanoparticles to Proteic Antigens Elicit Similar Humoral Immune Responses to Those Obtained with Alum. Journal of Nanoscience and Nanotechnology. 2017;17(1):846-52.
9. Li D, Fu D, Kang H, Rong G, Jin Z, Wang X, et al. Advances and Potential Applications of Chitosan Nanoparticles as a Delivery Carrier for the Mucosal Immunity of Vaccine. Current Drug Delivery. 2017;14(1):27-35.
10. des Rieux A, Fievez V, Garinot M, Schneider Y-J, Préat V. Nanoparticles as potential oral delivery systems of proteins and vaccines: A mechanistic approach. Journal of Controlled Release. 2006;116(1):1-27.
11. Amidi M, Romeijn SG, Borchard G, Junginger HE, Hennink WE, Jiskoot W. Preparation and characterization of protein-loaded N-trimethyl chitosan nanoparticles as nasal delivery system. Journal of Controlled Release. 2006;111(1-2):107-16.
12. Illum L, Jabbal-Gill I, Hinchcliffe M, Fisher AN, Davis SS. Chitosan as a novel nasal delivery system for vaccines. Advanced Drug Delivery Reviews. 2001;51(1-3):81-96.
13. Vila A, Sánchez A, Janes K, Behrens I, Kissel T, Jato JLV, et al. Low molecular weight chitosan nanoparticles as new carriers for nasal vaccine delivery in mice. European Journal of Pharmaceutics and Biopharmaceutics. 2004;57(1):123-31.
14. van der Lubben IM, Kersten G, Fretz MM, Beuvery C, Coos Verhoef J, Junginger HE. Chitosan microparticles for mucosal vaccination against diphtheria: oral and nasal efficacy studies in mice. Vaccine. 2003;21(13-14):1400-8.
15. Amidi M, Romeijn SG, Verhoef JC, Junginger HE, Bungener L, Huckriede A, et al. N-Trimethyl chitosan (TMC) nanoparticles loaded with influenza subunit antigen for intranasal vaccination: Biological properties and immunogenicity in a mouse model. Vaccine. 2007;25(1):144-53.
16. Murata Y, Toniwa S, Miyamoto E, Kawashima S. Preparation of alginate gel beads containing chitosan nicotinic acid salt and the functions. European Journal of Pharmaceutics and Biopharmaceutics. 1999;48(1):49-52.
17. Fröhlich E. The role of surface charge in cellular uptake and cytotoxicity of medical nanoparticles. International Journal of Nanomedicine. 2012:5577.
18. Lemarchand C, Gref R, Couvreur P. Polysaccharide-decorated nanoparticles. European Journal of Pharmaceutics and Biopharmaceutics. 2004;58(2):327-41.
19. World Health Organization, W., *WHO guidelines for the production, control and regulation of snake antivenom immunoglobulins.* Geneva: WHO, 2010. **134**.
20. Mohan, T., P. Verma, and D.N. Rao, *Novel adjuvants & delivery vehicles for vaccines development: a road ahead.* The Indian journal of medical research, 2013. **138**(5): p. 779.
21. Gomes A, Mohsen M, Bachmann M. Harnessing Nanoparticles for Immunomodulation and Vaccines. Vaccines. 2017;5(1):6.
22. Anandhakumar S, Krishnamoorthy G, Ramkumar KM, Raichur AM. Preparation of collagen peptide functionalized chitosan nanoparticles by ionic gelation method: An effective carrier system for encapsulation and release of doxorubicin for cancer drug delivery. Materials Science and Engineering: C. 2017;70:378-85.

23. Nguyen TV, Nguyen TTH, Wang S-L, Vo TPK, Nguyen AD. Preparation of chitosan nanoparticles by TPP ionic gelation combined with spray drying, and the antibacterial activity of chitosan nanoparticles and a chitosan nanoparticle–amoxicillin complex. Research on Chemical Intermediates. 2016;43(6):3527-37.
24. Esfandiarpour-Boroujeni S, Bagheri-Khoulenjani S, Mirzadeh H, Amanpour S. Fabrication and study of curcumin loaded nanoparticles based on folate-chitosan for breast cancer therapy application. Carbohydrate Polymers. 2017;168:14-21.
25. Orellano MS, Porporatto C, Silber JJ, Falcone RD, Correa NM. AOT reverse micelles as versatile reaction media for chitosan nanoparticles synthesis. Carbohydrate Polymers. 2017;171:85-93.
26. Zhang E, Xing R, Liu S, Li K, Qin Y, Yu H, et al. Comparison in docetaxel-loaded nanoparticles based on three different carboxymethyl chitosans. International Journal of Biological Macromolecules. 2017;101:1012-8.
27. Rezayat, M., *Preparation, characterization and stability investigation of chitosan nanoparticles loaded with the Echis carinatus snake venom as a novel delivery system.* Archives of Razi Institute, 2015. **70**(4): p. 269-277.
28. Mohammadpour Dounighi, N., et al., *Preparation and in vitro characterization of chitosan nanoparticles containing Mesobuthus eupeus scorpion venom as an antigen delivery system.* Journal of Venomous Animals and Toxins Including Tropical Diseases, 2012. **18**(1): p. 44-52.
29. Fissan H, Ristig S, Kaminski H, Asbach C, Epple M. Comparison of different characterization methods for nanoparticle dispersions before and after aerosolization. Analytical Methods. 2014;6(18):7324.
30. Tuoriniemi J, Johnsson A-CJH, Holmberg JP, Gustafsson S, Gallego-Urrea JA, Olsson E, et al. Intermethod comparison of the particle size distributions of colloidal silica nanoparticles. Science and Technology of Advanced Materials. 2014;15(3):035009.
31. Gan Q, Wang T. Chitosan nanoparticle as protein delivery carrier—Systematic examination of fabrication conditions for efficient loading and release. Colloids and Surfaces B: Biointerfaces. 2007;59(1):24-34.
32. Gan Q, Wang T, Cochrane C, McCarron P. Modulation of surface charge, particle size and morphological properties of chitosan–TPP nanoparticles intended for gene delivery. Colloids and Surfaces B: Biointerfaces. 2005;44(2-3):65-73.
33. Wu Y, Yang W, Wang C, Hu J, Fu S. Chitosan nanoparticles as a novel delivery system for ammonium glycyrrhizinate. International Journal of Pharmaceutics. 2005;295(1-2):235-45.
34. Zhang H-l, Wu S-h, Tao Y, Zang L-q, Su Z-q. Preparation and Characterization of Water-Soluble Chitosan Nanoparticles as Protein Delivery System. Journal of Nanomaterials. 2010;2010:1-5.
35. Dounighi, N.M., et al., *Preparation Of Chitosan Nanoparticles Containing Naja Naja Oxiana Snake Venom.* Archives of Pharmacy Practice, 2012. **3**(1): p. 11.
36. Xu Y, Du Y. Effect of molecular structure of chitosan on protein delivery properties of chitosan nanoparticles. International Journal of Pharmaceutics. 2003;250(1):215-26.
37. Jarudilokkul S, Tongthammachat A, Boonamnuayvittaya V. Preparation of chitosan nanoparticles for encapsulation and release of protein. Korean Journal of Chemical Engineering. 2011;28(5):1247-51.
38. Monzavi, S.M., B. Dadpour, and R. Afshari, *Snakebite management in Iran: Devising a protocol.* Journal of research in medical sciences: the official journal of Isfahan University of Medical Sciences, 2014. **19**(2): p. 153.
39. Mohammadpourdounighi N, Behfar A, Ezabadi A, Zolfagharian H, Heydari M. Preparation of chitosan nanoparticles containing Naja naja oxiana snake venom. Nanomedicine: Nanotechnology, Biology and Medicine. 2010;6(1):137-43.
40. Dounighi, N., et al., *Preparing and characterizing chitosan nanoparticles containing Hemiscorpius lepturus scorpion venom as an antigen delivery system.* Archives of Razi Institute, 2012. **67**(2): p. 145-153.
41. Zhou S, Deng X, Li X. Investigation on a novel core-coated microspheres protein delivery system. Journal of Controlled Release. 2001;75(1-2):27-36.
42. Dailey LA, Wittmar M, Kissel T. The role of branched polyesters and their modifications in the development of modern drug delivery vehicles. Journal of Controlled Release. 2005;101(1-3):137-49.