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Research Article

Preparation of Chitosan Nanoparticles as a Capable Carrier for Antigen Delivery and Antibody Production

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Background: Chitosan (CS) nanoparticles have attracted considerable attention as a non-viral and cationic carrier for delivery of therapeutic proteins and antigens and offer non-invasive routes of administration such as oral, nasal and ocular routes, and also show adjuvant characteristics for vaccines.

Objectives: Preparation and formulation of CS nanoparticles as a capable carrier with immunoadjuvant properties to enhance the bioavailability of antigen and produce antibody with high affinity.

Materials and Methods: CS nanoparticles were produced by ionic gelation process of sodium tripolyphosphate (TPP) with CS. Particle size and morphology of nanoparticles were determined using Dynamic Light Scattering (DLS) and Scanning Electron Microscopy (SEM) and also direct observation under light microscope. The influence of the initial BSA concentration and CS concentration on loading efficiency and release behavior was evaluated. The ε-toxin (derived from *Clostridium perfringens* type D) was loaded on CS nanoparticles and the complex was injected hypodermically into the rabbits for once. The anti ε-toxin antibody level in blood serum was evaluated using Dot Blot and ELISA methods.

Results: The CS nanoparticles in different groups have a particle diameter (Z-average) in approximate ranges of 200-400, 300-600, 450-800 nm and a positive Zeta potential (32.4 - 48.6 mv). Optimum loading efficiency was achieved for CS at a concentration of 0.5 $mg.mL^{-1}$ and TPP of 1.0 $mg.mL^{-1}$. The results showed that the toxin-CS complex produces antitoxin at levels more than twice as high the control.

Conclusion: The CS nanoparticles can be used as a good biodegradable carrier for protein and antigen delivery.

Keywords: Chitosan nanocarriers, High affinity antibody, Protein delivery, Tripolyphosphate

1. Background

The hydrophilic, nontoxic and natural nanoparticles have received much attention as carriers to delivery of therapeutic proteins and antigens (1). The advantages of nanoparticles over micro particles in drug delivery procedure are very significant, which is mainly due to the fact that their submicron size offers more bioavailability and biocompatibility (2-4). A fundamental requirement for nanoparticles used for humans or animals is that they have to degrade into molecules with no toxicity for the biological system (5). Nanoparticles offer non-invasive routes of administration such as oral, nasal and ocular routes, and also show adjuvant characteristics for vaccines (6, 7).

Over the last decades, Chitosan (CS) has attracted considerable attention as a pharmaceutical excipient and as a biomass in drug delivery systems. CS has shown favorable biocompatibility characteristics as well as the ability to increase membrane permeability, and can be degraded by lysozyme in serum (8). Furthermore, it possesses positive charge and exhibits absorption enhancing effects (6). From a drug delivery viewpoint, CS has many advantages, particularly for expanding micro/nano particulate systems, such as the capability for controlled release of active agents, avoidance of the use of hazardous organic solvents, and mucoadhesive characteristics which increases the residual time at the site of adsorption (6, 9-12). Sodium tripolyphosphate (TPP) is a nontoxic and multivalent anion. It can form gel by ionic interaction between positively charged amino groups of chitosan (CS has OH and groups that give rise to hydrogen bonding in aqueous solution) and negatively charged counter ions of TPP (13).

CS nanoparticles are attractive, non-viral and cationic carriers for delivery of peptides, proteins, and therapeutic antigens (12, 14-18).

2. Objectives

Our approach was to prepare and produce CS nanoparticles as a capable carrier with immunoadjuvant properties for antigen delivery and antibody production. Moreover, CS nanoparticles have potential capability to adsorb proteins on their surface which can deliver proteins to targeting sites and produce antibodies with high affinity to antigens.

3. Materials and Methods

Chitosan low-viscous from shrimp shell was purchased from Sigma - Aldrich (chemie GmbH, CH-9471 Buchs). Sodium tripolyphosphate anhydrous (MW=367.86 $g.moL^{-1}$ ($Na_5P_3O^{10}$) was purchased from Scharlau chemie S.A. As a model protein for determination of loading efficiency and controlled release, Bovine Serum Albumin (BSA) (Albumin fraction V) was purchased from Merck. Tween 80 as an emulsifier agent was purchased from Merck-Schuchardt. Coomassie Brilliant Blue G 250 for preparation of Bradford reagent was purchased from Merck. Purified ε-toxin was prepared from Razi Vaccine and Serum Research Institute, Mashhad, Iran. 2,2-azino-bis (3-ethylbenzothiazoline – 6 – sulfonic acid) - Liquid substrate system (ABTS) was purchased from sigma.

3.1. Preparation of CS Nanoparticles

CS solution was prepared by dissolving of low molecular weight CS powder at different concentrations (0.25, 0.5, 1.0, 1.5, 2.0 $mg.mL^{-1}$) in acetic aqueous solution. The acetic acid concentration in all preparations was 1.5 times higher than the CS concentration (2, 8). CS solution was then filtered by Whatman filter paper No.7 to remove the insoluble materials and then twice filtered by 0.22 μm filter to achieve solution of high homogeneity without any insoluble CS.

In order to prevent formation of particles with large diameter and avoid coagulation of nanoparticles, an emulsifier (Tween 80 at 0.005-0.02% v/v) was added to the CS solution. The aqueous solution of TPP at 1.0 $mg.mL^{-1}$ was added to the CS solution under constant magnetic stirring at ambient temperature. The adopted final volume ratio of TPP:CS in all cases was 2:5. The nanoparticles were separated from suspension by ultracentrifugation at 14000 x g at 14 °C for 20 min.

3.2. Preparation of BSA-Loaded CS Nanoparticles

After centrifuging, the separated nanoparticles from suspension were mixed with solutions containing BSA at predetermined concentrations. The mixed solutions were gently incubated and shaken for 0.5-1.0 h at ambient temperature to allow protein adsorption on the nanoparticles. This temperature isn't harmful to protein for short time. In this technique, protein loading is performed solely via adsorption on the surface of the nanoparticles. The mechanism of adsorption is the hydrogen bonds between negatively charged groups of protein molecules with the positively charged amine groups of chitosan nanoparticles surface.

3.3.Physicochemical Characterization of CS Nanoparticles

The particle size, zeta potential and polydispersity (size distribution) of CS nanoparticles were measured by Dynamic Light Scattering (DLS) using zeta sizer nano-zs 3000-HS (Malvern Instrument Ltd., Malvern, UK). Samples were diluted to appropriate concentrations with deionized water. Triplicate samples were evaluated and the arithmetic mean value of the three samples was adopted. The particle size distribution of

the nanoparticles is reported as a polydispersity index (PDI), ranging from 0 for an entire monodisperse up to 1 for a completely heterodisperse system.

Morphological characteristics of the nanoparticles were examined using Scanning Electron Microscopy (SEM). A droplet of the CS nanoparticles suspension was placed on an aluminium stub. After air-drying overnight at ambient temperature, the dried nanoparticles were coated with (Au-Pd) sputter coater device SC 7620 (England) and evaluated with a LEO 1450 VP scanning electron microscope (2.5 nm resolution and maximum voltage 35 kV, Germany).

3.4. Evaluation of Protein Loading Efficiency and Release Behavior

BSA-loaded CS nanoparticles were prepared in a 1.5 mL centrifugal microtube. Nanoparticles were separated from the suspension by ultracentrifugation at 14,000 x g at 14 °C for 20 min. The supernatant from the centrifuge was evaluated for protein determination by UV spectrophotometer at 595 nm using the Bradford Protein Assay. Bradford assay was used to measure the concentration of the total protein in solution based on the binding of coomassie Brilliant Blue G250 to the protein molecules. This method is fast, simple and sensitive and acts very specifically for proteins. The amount of protein bound to nanoparticles was calculated as the difference between the free protein in the supernatant and the initial total protein. The BSA loading efficiency (LE) was calculated by Eq. (1):

$$LE\ \% = (A-B)/A * 100 \quad (1)$$

where A is the total amount of BSA added to the nanoparticles, and B is the amount of non-bonded protein remaining in the supernatant.

To evaluate the release behavior, the nanoparticles that remained in the centrifugal tube were mixed and incubated with 1 mL PBS (pH 7.4).

At specified collection times, 100 µL test sample was taken from the supernatant and the amount of the released BSA from the nanoparticles was measured using Bradford Assay. The total released protein mass at time i was calculated by Eq. (2):

$$M_i = C_i V + \sum C_{i-1} V_t \quad (2)$$

where C_i is the concentration of protein in the release solution at time i, V is the total volume of release solution and V_t is the test sample volume (100 µL).

3.5. Immunization Study

Immunogenicity of ε-toxin loaded CS nanoparticles was evaluated using rabbits following hypodermically injection. 100 µL of CS nanoparticles was washed two times with PBS (pH 7.4) and supernatants were discarded. Then, 1 mL of the purified ε-toxin (50 $mg.mL^{-1}$) was incubated in CS nanoparticles and injected hypodermically in two conscious rabbits. 21 days after Injection, blood samples were taken from rabbits' hearts (1-2 mL). The collected blood samples were stored at 4 °C overnight and then centrifuged (3000 x g, 10 min) at room temperature. The resulting serum samples were maintained in freezer until analysis.

3.6. Evaluation of Antibody Production

The collected serum was tested by dot blot and ELISA for evaluation of antibody against ε-toxin. For ELISA survey, 150 µL of purified ε-toxin (5 $mg.mL^{-1}$) diluted in Tris buffer (20 mM) was added to microplates and incubated on a rotary shaker for 1.5 h at 37 °C .The wells were washed three times with PBS containing 0.05 % (v/v) Tween 80 (PBST). To minimize non-specific reactions, 200 µl of PBS containing 1 % (w/v) of BSA was added to the wells and incubated for 1 h at 37° C.

After three washes of the plate with PBST, various serial dilutions of test and control antiserum were added to the plates and incubated for 1 h at 37and washed. Then, 150 µL of anti-rabbit IgG peroxidase conjugate (diluted 1:1000 in PBS) was added to each well and incubated for another 1 h at 37 °C. The wells were washed and the reaction was developed with ABTS substrate, 150 µL of ABTS was added to each well. The following color development (15 min) plates were read at 405 nm on an ELISA reader (Biotek).

3.7. Statistical Analysis

Statistical analysis was performed with SPSS software (version 19.0). All the tests were run in triplicate (n=3). The results expressed as Mean ± SD. Statistical significance was determined by student's t-test. p values less than 0.05 were considered to be statistically significant.

4. Results

4.1. Effect of CS Concentration on CS Nanoparticles

The results showed that in higher CS concentrations (CS ≥ 2.0 mg.mL^{-1}) production of nanoparticles were extremely difficult. On the other hand, lower concentrations of CS (CS ≤ 0.25 mg.mL^{-1}) led to aggregation of products and formation of large diameter complexes. The best concentration range of CS nanoparticles production in this work was between 0.5-1.5 mg.mL^{-1} of CS and TPP concentration was adjusted to 1.0 mg.mL^{-1} for all.

4.2. In vitro Release of BSA from CS Nanoparticles

Figure 1 indicates that by increasing the CS concentration from 0.5 to 1.5 mg.mL^{-1}the release behavior of BSA from CS nanoparticles did not change noticeably ($P > 0.05$). The release percentage changed slightly in comparison to the conversion of CS concentration.

The release profiles are similar and show slow and controlled release behavior at different times ($P < 0.05$). All release profiles of the nanoparticles exhibit a burst release of about 45% in the first 24 h. After this time, a slow release of constant rate was observed which continued up to about 55% release after 96 h remaining in PBS. BSA release from nanoparticles after 24 h showed that the higher BSA initial loading concentration leads to greater release from nanoparticles (**Fig. 2**).

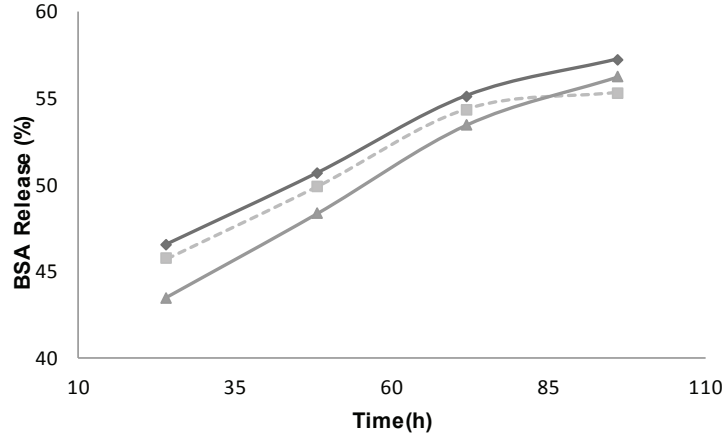

Figure 1. BSA release profile by time from CS nanoparticles at different concentrations of CS: (♦ = 0.5, ■ = 1.0, ▲= 1.5 mg.mL^{-1} ; BSA concentration: 1.0 mg.mL^{-1}

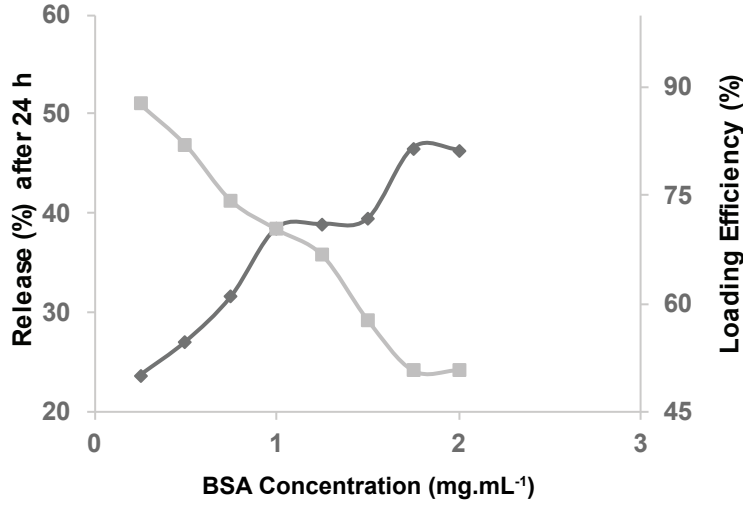

Figure 2. Influence of BSA initial concentration on loading efficiency (■ = LE %) and release behavior after 24 h (♦ = REL %) of CS nanoparticles. Particle preparation conditions: CS concentration: 0.5 mg.mL^{-1} , TPP concentration: 1.0 mg.mL^{-1} .

4.3. Characterization of CS Nanoparticles

Morphological characteristics and the surface morphology of the CS nanoparticles and BSA-loaded CS nanoparticles were examined using SEM technique. According to data analysis and SEM results, all the samples had nanometric sizes and spherical shapes. In this study, we measured the size and zeta potential of our best quality samples using dynamic light scattering (DLS) method. Samples had the mean size of less than 1 µm related to preparation conditions changing from 199.8 to 801.07 nm (**Fig. 3**). All the samples had zeta potential with positive charges in the range of 32.4 - 48.6 mv. Differences between size and morphology of CS nanoparticles and zeta potential values were due to various conditions in preparation methods such as distance between nozzle tip and liquid surface, volume rate and atomization pressure of TPP solution, reaction temperature and type of CS solution filtration. The effect of different parameters on the preparation of CS nanoparticles is listed in **Table 1**.

The particle size of BSA-loaded nanoparticles significantly increased in higher concentrations of BSA. **Figure 2** shows that the loading efficiency decreased from 87.72 to 50.78% by increasing the BSA initial concentration from 0.25 to 2.0 $mg.mL^{-1}$. Similar observations were also reported by others (19). Evaluation of the nanoparticles' size by DLS shows that the mean size of BSA-loaded CS nanoparticles is between 1300 - 1400 nm. This value is approximately twice as large the CS nanoparticles produced in this work. The shapes of the nanoparticles in SEM indicates that after bonding with BSA, the particles have larger mean diameter size and spherical shape with smooth surface and almost homogeneous structure (**Fig. 4**) (20).

4.4. Dot Blot and ELISA Analysis

A Dot Blotting test was used to determine the existence of IgG antibodies against ε-toxin in rabbits' serum after hypodermal administration. Dot blot results showed the presence of IgG against ε-toxin in rabbits' antiserum. A parallel ELISA test was used to determine the affinity of antibodies in rabbits' antiserum against ε-toxin. For this goal, a serial dilution of antiserum (1/1000,

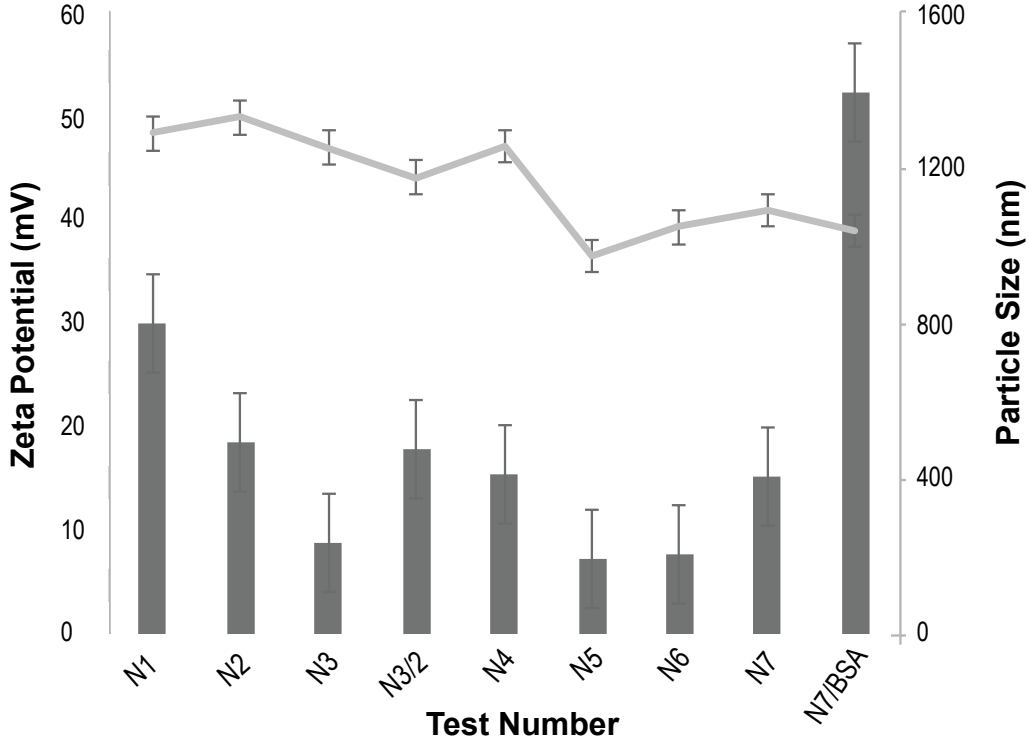

Figure 3. Mean Particle size (▬) and Zeta Potential (━) of CS nanoparticles Prepared via ionic gelation of CS and TPP, measured with DLS.

1/2000, 1/3000, 1/4000, 1/5000, 1/6000) was added to distinctive concentration of ε-toxin 5 mg.mL^{-1} in constant volume (150 µL) following an ELISA method and the amount of the reacted antibodies with antigens was characterized using ABTS substrate on an ELISA reader at 405 nm. Our results indicated that 1/3000 dilution of antibody is saturated and in this dilution all the antibodies reacted with ε-toxin (data not shown). In contrast, the same O.D was detected at 1/1000 dilution rate for the control. An ELISA test was performed to determine the amount of antibodies produced against ε-toxin. Our results showed that the ε-toxin bound CS nanoparticles produce antitoxin more than twice of the control (**Fig. 5**).

Table 1. Different physicochemical parameters that affected on preparation of CS nanoparticles.

Sample no.	CS (mg.mL^{-1})	TPP (mg.mL^{-1})	TPP/CS (v/v)	Tween80%(v/v)	Atomization Pressure (bar)	*Remain Time (day)
N1	0.5	1.0	2/5	0.02	1-1.5	11
N2	0.5	1.0	1/5	0.02	0.5 - 1	12
N3	0.5	1.0	2/5	0.02	2	86
N3/2	0.5	1.0	2/5	0.02	2	86
N4	0.5	1.0	1/2	0.01	2	69
N5	0.5	1.0	1/4	0.005	0.5-1	38
N6	0.5	1.0	2/5	0.5	2	89
N7	1.0	1.0	2/5	0.005	0.5 - 1	47
N7/BSA	1.0	1.0	2/5	0.005	0.5 - 1	57

*The remain time is the time that samples have been maintained in the refrigerator after producing process until analyzing with DLS.

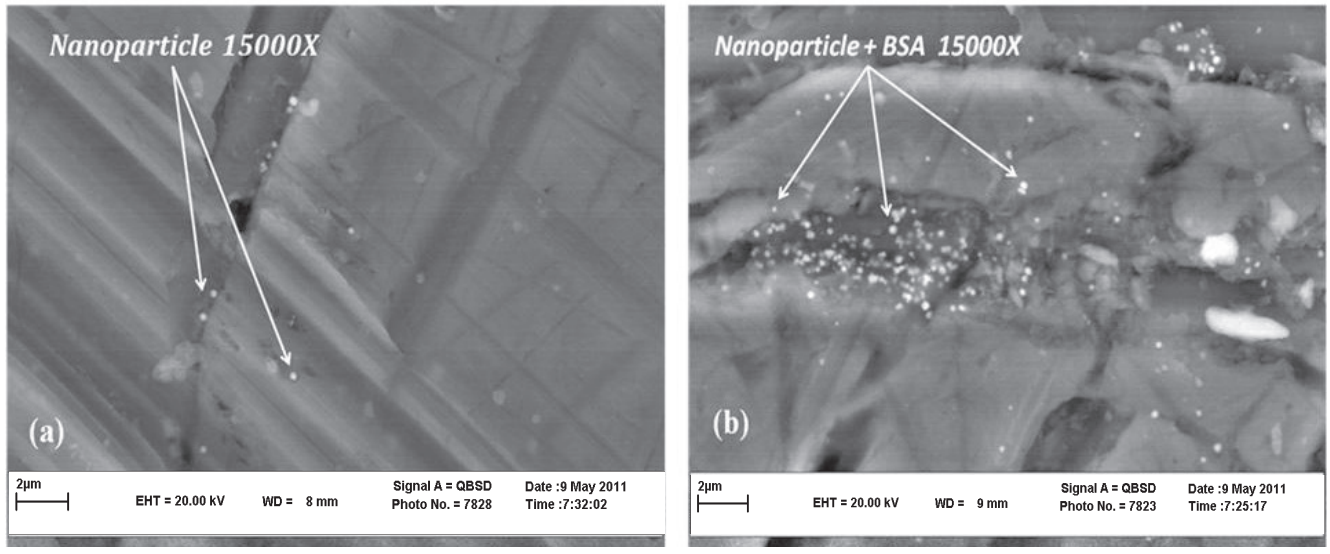

Figure 4. Scanning electron microscopy (SEM) images of CS nanoparticles; **(a)** non-loaded nanoparticles, **(b)** BSA-loaded nanoparticles. Particle preparation conditions: CS concentration: 0.5 mg.mL^{-1}, BSA concentration: 1.0 mg.mL^{-1} , TPP concentration: 1.0 mg.mL^{-1}, pH 4.0.

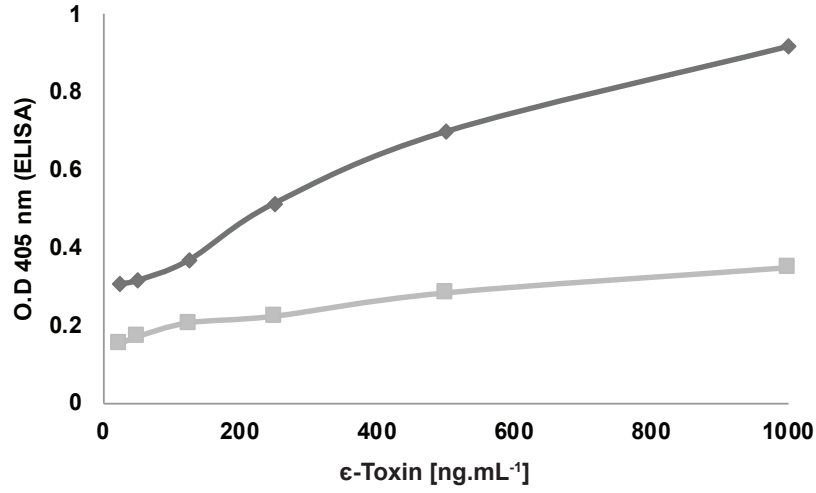

Figure 5. Assay of ε-toxin and nanoparticles bound ε-toxin by ELISA (■ = ε-toxin, ♦ = ε-toxin loaded nanoparticles).

5. Discussion

The main goal of this work was to investigate the potential capability of low viscous CS nanoparticles for antigen delivery and antibody production. We evaluated the effect of CS concentration on loading efficiency and release behavior.

The observations indicated that the ionic gelation leads to form some opaline colloidal suspension at the final optimization. Also, it was found that lower viscosity of CS (low MW) with lower concentration (such as 0.5-1.5 mg.mL^{-1} promotes better gelation. Similar observation was reported by Xu *et al*. (2). They showed that CS concentration of the range 1-3 mg.mL^{-1} results in the formation of CS nanoparticles and by decreasing CS concentration to 0. mg.mL^{-1}, aggregates with large diameters forms. Our results indicated that 0.5 mg.mL^{-1} of CS and 1.0 mg.mL^{-1} of TPP leads to the highest loading efficiency and slow release rate of BSA. Similar results were reported by others (1, 2, 8, 11, 12, 19-21) for the encapsulation of protein in CS nanoparticles. They all showed that concentration of CS and TPP were important in formation of nanoparticles. Method of preparation and mixture of these two in proper buffer system is also critical. Although in most of the previous works, the adopted CS concentration for the production of encapsulated CS nanoparticles was in the range of 1-3 mg.mL^{-1}, but in this work a lower concentration of 0.5 mg.mL^{-1} CS was used to produce CS nanoparticles. In this concentration, all the nanoparticles had uniform structure with spherical shape and smooth surface with potential for use in many CS based systems. Formations of CS nanoparticles with a good morphological structure and proper porosity is important for the creation of sufficient space for binding of proteins. According to various reports (1, 2, 8, 11, 12, 19-23) uniform structure of the particles increases the capacity of the system for absorption of protein and increases the surface area of nanoparticles. Protein release behavior from nanoparticles is directly associated with initial protein loading concentration. It means that at higher BSA loading concentrations, binding of BSA molecules on CS surface molecules is weaker. This could be due to the protein molecules competing for multiple hydrogen bonds with the amine groups and also due to spatial exclusions (20). With respect to the SEM results and size of the particles, N7/BSA sample in comparison with N7 (without BSA bonding on its surface) has higher mean diameter. It means that loading CS nanoparticle with BSA results in larger particle diameter size and lowers zeta potential and surface charge density. This result shows that when CS nanoparticles were washed with distilled water after primary ultracentrifugation causes an adherence of CS nanoparticles together and a large mean particle size (1000-1100 nm) formed but addition of BSA or other protein to washed CS nanoparticles helps separating the nanoparticles and binding to BSA. In this step, the size of particles increases nearly to 1400 nm (data not

shown). The obtained nanoparticles in suspension can absorb various types of proteins on its surface due to the hydrophilicity and high positive surface charge. Our results indicated that nanoparticles with lower size could absorb higher percentage of protein compared to microparticles. Similar studies were also done by other researchers and they showed that the percentage of protein binding to the nano and microparticles were different (1, 2, 11, 12, 14, 19, 21, 22, 24). Xu and Du showed that the structure and molecular parameters of chitosan nanoparticles play an important role in protein delivery (2, 25). Altering concentration of BSA from 0.2 to 2 $mg.mL^{-1}$ and chitosan from 3 to 1 $mg.mL^{-1}$ significantly enhance the encapsulation capacity of BSA. In this regard, Amidi *et al*. (1) suggested that small size stable TMC (N-trimethyl chitosan) particles with a narrow size distribution have an excellent loading capacity for proteins, and a positive surface charge, suitable for attachment to nasal mucosa. According to the Results of this study, the amount of IgG detected in antiserum of the injected animal with CS nanoparticles was 2 to 3 times more than that of the others. There are different reports that CS nanoparticles elicited and enhanced humoral immunogenic response (IgG titers) as compared to fluid vaccines (15, 26). Based on the ELISA analysis, suggesting nanoparticles of CS could be a good carrier system for transformation of toxin and slow release of antigen in the host.

6. Conclusion

The results indicated that CS micro and nanoparticles could be used as a good delivery system for transportation of antigens with negative charge and enhance the immune system for antibody production and protection induction. This ability of CS nanoparticles as adjuvants to motivate significant responses can be due to chitosan's specific properties such as being considered as a safe material as it is a natural, biocompatible, biodegradable, bioadhesive and water-soluble polymer, easy to prepare, suitable for a broad category of antigens and drugs. In addition, its nano scale size facilitates the drug uptake through the cell membrane. Also, chitosan provides absorption promoting effect that prolongs the contact time between substrate and cell membrane. We suggest CS nanoparticles as a good candidate for replacement with most of oil-based adjuvants for delivery of antigens.

References

1. 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. *J Cont Rel*. 2006;**111**:107-116. doi:10.1016/j.jconrel.2005.11.014
2. Xu Y, Du Y. Effect of molecular structure of chitosan on protein delivery properties of chitosan nanoparticles. *Int J Pharm*. 2003;**250**:215-226.
3. Akerele G, Ramadan N, Renu S, Renukaradhya GJ, Shanmugasundaram R, Selvaraj RK. *In vitro* characterization and immunogenicity of chitosan nanoparticles loaded with native and inactivated extracellular proteins from a field strain of *Clostridium perfringens* associated with necrotic enteritis. *Vet Immunol Immunopathol*. 2020;**224**:110059. doi: 10.1016/j.vetimm.2020.110059
4. Almasian P, Amani J, Arani FB, Nazarian S, Kazemi R, Tabrizi NM. Preparation of chitosan nanoparticle containing recombinant StxB antigen of EHEC and evaluation its immunogenicity in BALB/c mice. *Iran J Microbiol*. 2018;**10**(6):361-370. doi:10.1016/j.micpath.2018.08.037
5. Lopez-Leon T, Carvalho ELS, Seijo B, Ortega-Vinuesa JL, Bastos-Gonzalez D. Physicochemical characterization of chitosan nanoparticles: electrokinetic and stability behavior. *J Coll Int Sci*. 2005;**283**:344-351. doi:10.1016/j.jcis.2004.08.186
6. Tiyaboonchai W. Chitosan Nanoparticles : A Promising System for Drug Delivery. *Nar Uni J*. 2003;**11**(3):51-66.
7. Ghaffari Marandi BH, Zolfaghari MR, Kazemi R, Motamedi MJ, Amani J. Immunization against Vibrio cholerae, ETEC, and EHEC with chitosan nanoparticle containing LSC chimeric protein. *Microb Pathog*. 2019;**134**:103600. doi:10.1016/j.micpath.2019.103600
8. Wu Y, Yang W, Wang C, Hu J, Fu S. Chitosan nanoparticles as a novel delivery system for ammonium glycyrrhizinate. *Int J Pharm*. 2005;**295**:235-245. doi:10.1016/j.ijpharm.2005.01.042
9. Cui F, Qian F, Yin C. Preparation and characterization of mucoadhesive polymer-coated nanoparticles. *Int J Pharm*. 2006;**316**:154 -161.
10. Kiang T, Wen J, Lim HW, Leong KW. The effect of the degree of chitosan deacetylation on the efficiency of gene transfection. *Biomaterials*. 2004;**25**:5293–5301. doi:10.1016/j.biomaterials.2003.12.036
11. Mohammadpourdounighi N, Behfar A, Ezabadi A, Zolfagharian H, Heydari M. Preparation of chitosan nanoparticles containing Naja naja oxiana snake venom. *Nanomed Nanotech Bio Med*. 2009;**XX**. doi:10.1016/j.nano.2009.06.002
12. Xu Y, Du Y, Huang R, Gao L. Preparation and modification of N-(2-hydroxyl) propyl-3-trimethyl ammonium chitosan chloride nanoparticle as a protein carrier. *Biomaterials*. 2003;**24**:5015–5022. doi:10.1016/s0142-9612(03)00408-3
13. Ko JA, Park HJ, Hwang SJ, Park JB, Lee JS. Preparation and characterization of chitosan microparticles intended for controlled drug delivery. *Int J Pharm*. 2002;**249**:165-174. doi:10.1016/s0378-5173(02)00487-8
14. Aktas Y, Andrieux K, Alonso MJ, Calvo P, Neslihan G¨ursoy R, Couvreur P, *et al*. Preparation and *in vitro* evaluation of chitosan

nanoparticles containing a caspase inhibitor. *Int J Pharm.* 2005;**298**:378–383. doi:10.1016/j.ijpharm.2005.03.027

15. Vila A, Sanchez A, Janes K, Behrens I, Kissel T, Vila Jato JL, *et al*. Low molecular weight chitosan nanoparticles as new carriers for nasal vaccine delivery in mice. *Eur J Pharm Biopharm*. 2004;**57**:123–131. doi:10.1016/j.ejpb.2003.09.006
16. Shakeran Z, Keyhanfar M, Varshosaz J, Sutherland DS. Biodegradable nanocarriers based on chitosan-modified mesoporous silica nanoparticles for delivery of methotrexate for application in breast cancer treatment. *Mater Sci Eng C Mater Biol Appl.* 2021;**118**:111526. doi:10.1016/j.msec.2020.111526
17. Fabregas A, Minarro M, Garcia-Montoya E, Perez-Lozano P, Carrillo C, Sarrate R, *et al*. Impact of physical parameters on particle size and reaction yield when using the ionic gelation method to obtain cationic polymeric chitosan-tripolyphosphate nanoparticles. *Int J Pharm*. 2013;**446**(1-2):199-204. doi: 10.1016/j.ijpharm.2013.02.015
18. Yuan X, Shah BA, Kotadia NK, Li J, Gu H, Wu Z. The development and mechanism studies of cationic chitosan-modified biodegradable PLGA nanoparticles for efficient siRNA drug delivery. *Pharm Res.* 2010;**27**(7):1285-1295. doi:10.1007/s11095-010-0103-0
19. Chun W, Xiong F, LianSheng Y. Water-soluble chitosan nanoparticles as a novel carrier system for protein delivery. *Chin Sci Bull*. 2007;**52**:883-889.
20. Gan Q, Wang T. Chitosan nanoparticle as protein delivery carrier—Systematic examination of fabrication conditions for efficient loading and release. *Coll Surf B: Bioint.* 2007;**59**:24–34. doi:10.1016/j.colsurfb.2007.04.009
21. 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. *Coll Surf B: Bioint*. 2005;**44**:65-73. doi:10.1016/j.colsurfb.2005.06.001
22. Bayat A, Dorkoosh FA, Dehpour AR, Moezi L, Larijani B, Junginger HE, *et al*. Nanoparticles of quaternized chitosan derivatives as a carrier for colon delivery of insulin: Ex vivo and in vivo studies. *Int J Pharm.* 2008;**356**:259-266. doi:10.1016/j.ijpharm.2007.12.037
23. Zhang S, Huang S, Lu L, Song X, Li P, Wang F. Curdlan sulfate-O-linked quaternized chitosan nanoparticles: potential adjuvants to improve the immunogenicity of exogenous antigens via intranasal vaccination. *Int J Nanomedicine.* 2018;**13**:2377-2394. doi:10.2147/ijn.s158536
24. Janes KA, Alonso MJ. Depolymerized Chitosan Nanoparticles for Protein Delivery: Preparation and Characterization. *J App Pol Sci.* 2003;**88**:2769-2776. doi:10.1002/app.12016
25. Cai Y, Lapitsky Y. Biomolecular uptake effects on chitosan/tripolyphosphate micro- and nanoparticle stability. *Colloids Surf B Biointerfaces.* 2020;**193**:111081. doi:10.1016/j.colsurfb.2020.111081
26. Rezaei Mokarram A, Alonso MJ. Preparation and evaluation of chitosan nanoparticles containing Diphtheria toxoid as new carriers for nasal vaccine delivery in mice. *Arch Razi Ins.* 2006;**61**:13-25.