Original Article

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Preparation and Evaluation of Rivax Protein Loading in Chitosan Nanoparticles

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ABSTRACT

BACKGROUND AND OBJECTIVE: Ricin toxin is a heterodimer glycoprotein which, due to its high toxicity, is used as a bioterrorism agent. Immunogenicity studies against ricin are now focused on two subunit vaccine candidates, including RiVax and RVEc. These studies have examined the vaccine candidate immunization as an alone and in combination with adjuvant, however, there is not a published study on the immunogenicity evaluation of the candidate vaccine through the delivery by nanoparticles. The aim of this study was preparation and evaluation of RiVax recombinant vaccine-loading in chitosan nanoparticles.

METHODS: In this experimental study, After transferring the RiVax gene to the bacterium, inducing the expression and purification of the RiVax protein by affinity chromatography column, the RiVax protein was loaded with Ionic Gelation method in chitosan nanoparticles. Then, the properties of nanoparticles including size, morphology, loading percentage and release pattern of RiVax protein from nanoparticles and stability of this protein during acidic loading conditions in nanoparticles by SDS-PAGE were evaluated. Also, Immunization study were performed on 3 mouse groups (n=4/group) by RiVax protein, Nanoparticles containing protein and phosphate buffer.

FINDING: The results of this study showed that the nanoparticles containing protein had a size of 178 nm and a Zeta potential of +27.8 MV and a polydispersity index of 0.193. Also, according to SDS-PAGE results, it was found that the RiVax recombinant protein was denatured during the process of preparing the chitosan nanoparticles.

CONCLUSION: The results of this study showed that the RiVax protein has been unstable in acidic conditions for the production of chitosan nanoparticles and Ionic Gelation method is not suitable for loading this protein in chitosan nanoparticles.

KEY WORDS: *Chitosan, Nanoparticles, RiVax, Vaccine.*

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Introduction

Ricin is a toxic protein found in castor plant seeds. Ricin is a lectin glycoprotein with a molecular weight of 60-65 kDa and consists of two chains A and B, joined together by a disulfide bond (2, 1). The chain B facilitates the entry of the toxin into cytosol, and the A chain with molecular weight of 32 kDa, prevents protein synthesis by inactivating eukaryotic ribosomes, which ultimately leads to cell death (4, 3). Poisoning with Ricin can be by oral, inhalation, or injectable as well as from the eye and skin (1). There is currently no specific treatment for Ricin poisoning and, according to the signs of the disease, treatment is predominantly symptomatic (5). Animal studies show that by inactive or active immunization (by vaccine), it can be protected from poisoning with ricin (6). Smallshaw and colleagues investigated mutant recombinant A chain subunit with the aim of producing a Non-toxic and safe vaccine, and eventually introduced a recombinant vaccine candidate called RiVax. This protein has two mutations in the sequence, including tyrosine 80 to alanine (a place for the application of ribosomal toxicity) and valine 76 to methionine (a vascular leak syndrome motif). So far, various studies have been done on the stability and immunogenicity of RiVax and its various methods of administration to the body. Immunization tests for this vaccine candidate have also been studied with the adjuvant or alone on animals (11-7), but for administration of the vaccine to human, there is a need for alternative adjuvants and novel delivery systems.

However, so far, new delivery systems such as nanoparticles have not been used to investigate the improvement of immunization properties of this protein. One of the polymers that is widely used today for the production of biocompatible and biodegradable nanoparticles is chitosan, which is a good option for the transmission of radiopharmaceuticals, genes, proteins and peptides. Nowadays, chitosan nanoparticles are used for ophthalmic, respiratory, oral cavity, gastrointestinal and vaginal routes for prescribing drugs and vaccines (13, 12). The purpose of this study was to assess and evaluate the loading of recombinant vaccine RiVax in chitosan nanoparticles.

Methods

In this experimental study, after approval by the Ethics Committee of Imam Hossein University (AS) with code IR.IHU.REC.1396.1805, E. coli BL21-DE3 was used and its growth was carried out using LB culture medium and agar. Chemicals, kits and molecular markers were provided by Merck, Sinagen, Kiagen and Fermentas. Polymer chitosan with an average molecular from Sigma-Aldrich, Sodium Tripolyphosphate from Scharlau company and the synthetic gene of RiVax with two mutations in the A Ricin chain was provided by the Department of Biology at Imam Hussein University. To prepare the nanoparticle image, we used an SEM electron microscope manufactured by KYKY company, and we used the Malvern DLS device made in England to determine the size and Zeta potential of the nanoparticle.

Preparation of RiVax Recombinant Protein: After preparation of susceptible cells of E. coli BL21 (DE3), the synthetic gene containing RiVax protein was transformed into susceptible cells by heat shock method and induced the expression of RiVax gene at 30 ° C from isopropyl-Bd thiogalactopyranoside (IPTG) with a final concentration of 1 mM. Also, after lysis of bacteria and evaluation of the expression, Ni-NTA column with imidazole concentration gradient was used to purify the RiVax protein.

Preparation of chitosan nanoparticles containing RiVax protein: chitosan nanoparticles were prepared by ionization method. A solution with a concentration of 2 mg / ml of chitosan was prepared in acetic acid 2% w/v (pH = 4/5) and also a Sodium Tripolyphosphate solution at a concentration of 1 mg/ml was prepared. 5 ml of Sodium Tripolyphosphate solution as drip form was added to the 7.5-mL chitosan solution containing 1mg of recombinant protein while mounted on a magnetic stirrer at a constant speed. Finally, the solution was stirred for 30 minutes. After the reaction was completed, the solution containing nanoparticles at 4 ° C and in glycerol substrate was centrifuged for 30 minutes at a rate of 13500 rpm.

Physical and chemical characterization of nanoparticles by dynamic light scattering (DLS): To determine the size, dispersion index and zeta potential of produced nanoparticles, DLS was used and measurement was performed at 25 ° C.

Morphologic study of nanoparticles by electron microscope: SEM electron microscope was used to study the properties of nanoparticles. After the preparation of the chitosan nanoparticles, some of it was placed on a glass surface and dried at room temperature. The specimens were then coated with a thin layer of gold. Finally, the specimens were evaluated by a microscope.

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Indirect investigating the weight output and protein retention in nanoparticles: After the nanoparticles containing the antigen were made in optimal conditions, the resulting suspension was kept at 4 ° C for 30 minutes and was centrifuged at a rate of 13500 rpm, supernatant solution was collected. Then, by measuring the amount of free protein in the solution by the Bradford method, the amount of antigen enclosed in the nanoparticles was obtained and, by placing in the corresponding formulas, the efficiency and retention capacity of the nanoparticles were calculated. The weight output of combined nanoparticles was also calculated after freezing/drying, weighing. This work was done for both BSA-containing nanoparticles and nanoparticles containing recombinant RiVax protein (15, 14).

Exogenous release of antigen from chitosan nanoparticles: For the release of recombinant protein from the chitosan nanoparticles, a sample of nanoparticles at volume of 500 uL of SBF (Simulated Body Fluid) solution was poured into a 2 milliliter micro tubes and dispersed by stirring. Then the sample was incubated at 37 ° C in an incubator shaker at 200 rpm and its release was monitored for 192 hours. At each sampling time, the samples were centrifuged at 13500 rpm for 10 minutes and 500 µl of supernatant solution was removed and replaced with fresh buffer and incubated in a shaker. Then, using the three times Bradford method, the protein concentration in the sample was determined and the cumulative percentage of the released protein from the nanoparticles was plotted for the determined duration.

Investigation of the stability and shelf life of recombinant protein during preparation of chitosan nanoparticles: To investigate the possible effect of the chitosan nanoparticle production method on the stability and shelf life of recombinant protein, this protein was investigated in three conditions. In the first condition, the recombinant protein was added to a solution of 2 m/ml of chitosan in acetic acid 2 wt% (pH=4.5) and without addition of TPP (to prevent the production of nanoparticles and protein retention) and was stirred. In the second condition, the same conditions of the first state were performed without mixing, and in the third condition, the recombinant protein was mixed in aqueous solution without acetic acid. Finally, after 1 hour, the above solutions were injected into the SDS-PAGE gel wells and electrophoresed with a flow rate of 20 mA.

In-vitro studies of nanoparticles on the animal model: For the purpose of antibody production and evaluation of immune response, rats from the Razi Vaccine and Serum Institute were prepared. For evaluation of immunization, three groups of 4 rats weighing 25-20 g were used. At all stages, the work done on animals was in accordance with the recommended rules for working with laboratory animals. administration was performed in two days in the first days (D0) and fourteenth (D14), respectively. In the first group, the chitosan nanoparticles loaded with the recombinant protein and the second group received the recombinant protein alone. PBS was injected to the third group as control. Blood samples were collected one week after the second injection (D21) and after serum isolation, IgG antibody titer was measured by indirect ELISA method. Finally, statistical analysis of the samples was performed using Statistica software (Duncan test) and $p < 0.05$ was considered significant.

Results

Investigation of RiVax Protein Purification: After passing the bacterial lysis solution from the Ni-NTA column and washing with the imidazole concentration gradient, a purified protein band was observed in a 250 mill molar imidazole rinse buffer, which had a good purity (Fig. 1).

Physicochemical Properties of Nanoparticles by DLS: The results of the DLS showed that the protein-free nanoparticles had a size of 264 nm and PDI = 0.316 (Fig. 2a), and nanoparticles containing protein had a size of 178 nm and PDI= 0.193 (Fig. 2b) Also, the average zeta potential of the free chitosan nanoparticles or containing protein was 26+ and +27.8 MV, respectively (Fig. 3).

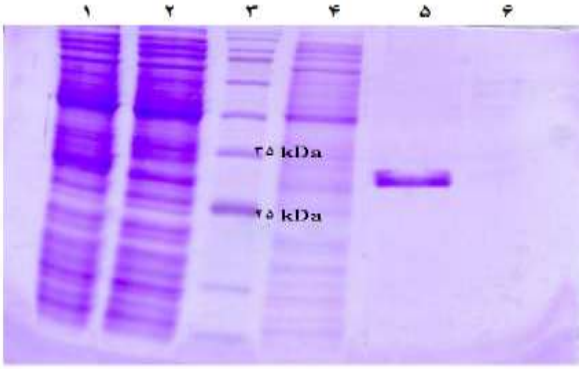

Figure 1. The result of expression and purification of RiVax protein on the SDS-PAGE gel. 1: Sample induced before crossing the Ni-NTA column. 2: Sample induced after crossing the column. 3: Molecular protein marker. 4: First rinse buffer (Imidazole 40mM). 5: Second rinse buffer (250 mM imidazole). 6: MES buffer.

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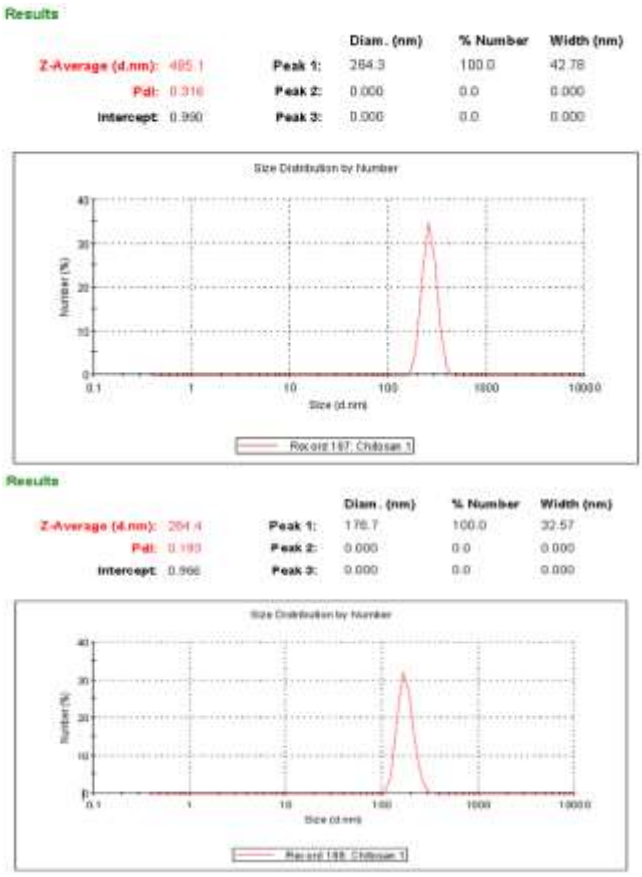

Figure 2. Distribution of the size of the chitosan nanoparticles, a) protein-free nanoparticles, b) nanoparticles containing protein in optimal conditions

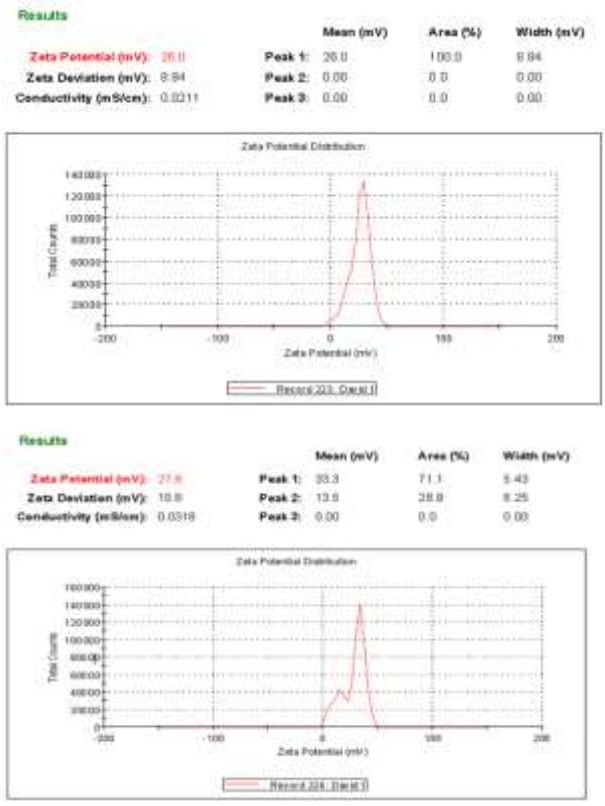

Figure 3. Zeta potential of chitosan nanoparticles, a) protein-free nanoparticles, b) nanoparticles containing protein in optimal conditions

Investigating the morphology of nanoparticles by electron microscopy: Electron microscopic images showed the spherical surface of the nanoparticles obtained from the production process in optimal conditions. These images also confirmed the results of the DLS device for the size distribution of nanoparticles (Fig. 4).

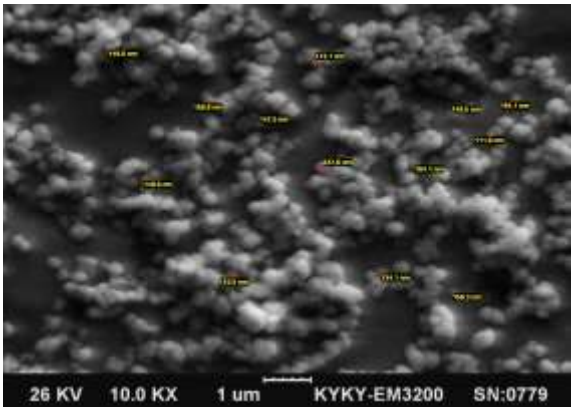

Figure 4. SEM electron microscope image of chitosan nanoparticles

Investigating the weight output and protein retention in nanoparticles indirectly: Weight output, retention efficiency, and RiVax protein retention capacity in chitosan nanoparticles were 59.5%, 100% and 8%, respectively, and for BSA as control were respectively, 57.2, 63/3% and 5.2%, respectively.

Investigating the exogenous protein release from chitosan nanoparticles: The results of analysis of the exogenous release of recombinant protein from chitosan nanoparticles showed that only about 2% of the protein was released in 192 hours, which is contrary to the expected release properties of chitosan nanoparticles (Fig. 5).

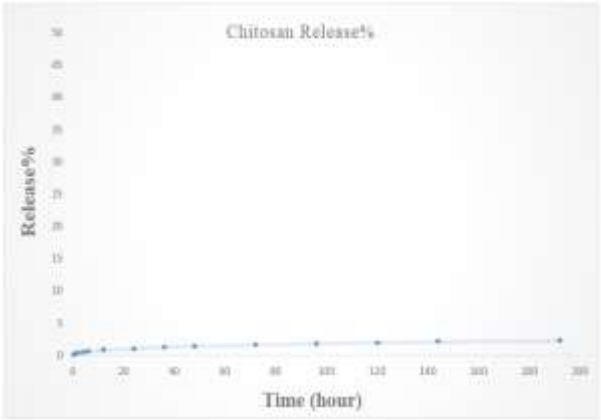

Figure 5. Cumulative percent of release of antigen from chitosan nanoparticles

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Results of the study of recombinant protein stability during preparation of chitosan nanoparticles: In the analysis of the SDS-PAGE results (Fig. 6), no bands were obtained from the first case, in which the recombinant protein dissolved in a chitosan solution with pH = 4.5 stirred for one hour, but bond of protein dissolved in a solution of chitosan with pH= 4.5 without mixing (second state) and the protein dissolved in water (third state) were observed in the gel.

The results of in vitro studies of nanoparticles on the animal model: The highest antibody titer was related to the protein alone group (2.606 ± 0.082) which was significantly different compared with that of the protein-containing nanoparticles (0.367 ± 0.005) and the control group ($p < 0.01$). Also, there was no statistically significant difference between the control samples and the loaded nanoparticles (Fig. 7)

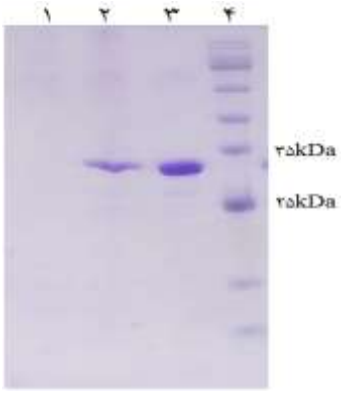

Figure 6 Electrophoresis pattern of evaluation of the stability and durability of recombinant protein during the preparation of chitosan nanoparticles. 1: Protein dissolved in a chitosan solution with pH = 4.5, which has been stirred for one hour (first mode). 2: Protein dissolved in a chitosan solution with pH = 4.5 without stirring (second mode). 3: Solubilized protein in water that has been stirred for one hour (third state). 4: Molecular protein marker.

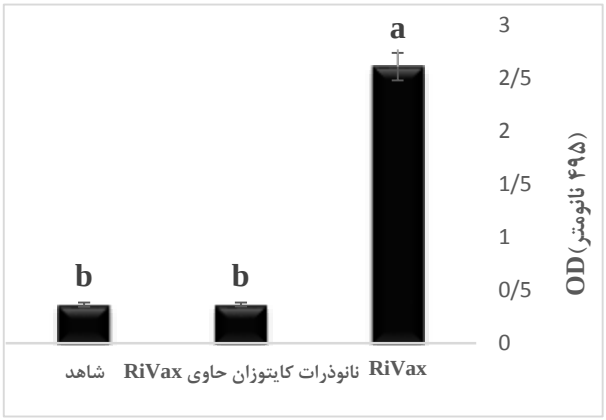

Figure 7. Comparison of the mean IgG titer in the serum of immunized mice from the injected route (non-identical letters indicate significant changes).

Discussion

The results of this study showed that despite the production of the nanoparticles in terms of physicochemical properties, the recombinant protein was completely degraded during the stages of preparation of chitosan nanoparticles and therefore loading of recombinant RiVax protein in chitosan nanoparticles was not possible due to the acidic conditions of the environment. The results of in vitro and in vivo studies of nanoparticles containing recombinant RiVax protein have also confirmed this results.

One of the common methods for production of chitosan nanoparticles is the ion gelation method used in this study. In this method, the chitosan become polycationic in acidic environments and can be attached to negative charge groups such as sulfate and phosphate. Sodium tripolyphosphate is a poly-anion that can be linked to the polymer by electrostatic forces between their phosphate groups and the amine groups of the chitosan. In this study, the best conditions for production of chitosan nanoparticles were considered to be ratio of 1 to 3 TPP solution to chitosan and pH 4.5 to dissolve chitosan. In the present study, the average size of chitosan nanoparticles without and with protein was 264 and 178 nm, respectively. In a study carried out by Bagheripour et al., after loading of the recombinant neurotoxin botulinum protein, chitosan nanoparticles containing protein were produced in the range of 285 nm (16). In another study by Lee et al., chitosan nanoparticles with a size of 150 to 250 nm were produced (17). The above results indicate that the size of the chitosan nanoparticles is within the same range as compared to other studies, and minor differences can be due to small variations in the production method and the various ratios of the materials. The results of the SEM measurements of nanoparticle size were also confirmed by the DLS results. Here, it is important to note that the size of the nanoparticle containing antigen is a determining factor in its absorption and bioavailability, and particles smaller than 2 micrometers have the ability to pass through mesenteric lymph nodes through the peyers plaque (18). In this research, the zeta potential of chitosan-containing protein and chitosan-free protein was +27.8 and 26+, respectively, and was consistent with the results of Amidi et al., which produced 150 to 300 nm chitosan nanoparticles with zeta potential of 16+ to +30 mV (19). The particle zeta potential is an important factor for their stability in the solution, and the chitosan particles with a higher zeta potential will have greater stability in the solution (20).

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Mohammadpour and colleagues in their research showed that the zeta potential of the chitosan nanoparticles decreases after loading with the protein (14). Hosseinzadeh and colleagues also showed that increasing the concentration of chitosan solution increases the size of the nanoparticles and its zeta potential. Also, with increasing TPP concentration, the size of nanoparticles is increased, but its zeta potential decreases (21). Overall, in the present study, by observing changes in the zeta potential in nanoparticles, it can be deduced that significant changes in the zeta potential have not occurred between antigen-free nanoparticles and antigen-bearing nanoparticles, and in both cases, the nanoparticles are in a stable range that prevents nanoparticles from joining together and forming particles that are glued together.

The present study was also used to investigate the retention efficiency and retention capacity of nanoparticles indirectly by investigating the protein concentration in the solution obtained from nanoparticle treatments, which results in 100% protein uptake in chitosan nanoparticles. However, when analyzing protein release from nanoparticles, the obtained pattern showed a very low protein release during the time period, while according to similar studies, most proteins were released from the chitosan nanoparticles during 7 days (22). According to these results, the possibility of denaturation of protein in the production of chitosan nanoparticles was considered. By designing a test and investigating the stability and shelf-life of RiVax protein in various acid and water conditions, it was found that the RiVax protein was strongly sensitive to acidic conditions and therefore during the production of chitosan nanoparticles, the protein is degraded. Therefore, contrary to the results of the retention rate, however, no protein was absorbed by the nanoparticles, which was confirmed by the release of the chitosan nanoparticles. Moreover, in the final stage, mouse groups were immunized alone by proteins and nanoparticles containing proteins, and the antibody titers in these mice also confirmed the degradation of the recombinant RiVax protein during the loading steps in the nanoparticles. In this regard, studies have shown that RiVax protein is a sensitive and unstable protein that is degraded in solutions that are not compatible with its properties (23).

Based on the results of this study, RiVax protein is slightly stable in acidic condition of production of chitosan nanoparticles, and other methods for loading this protein into nanoparticles should be used. In general, it can be concluded that the study of the retention efficiency and the retention capacity of nanoparticles indirectly does not confirm the definitive confirmation of the loading of the protein in the nanoparticles and requires a complementary method to confirm the shelf-life and stability of proteins during loading in the nanoparticles. In this study, a simple method for confirming the stability of proteins during loading in the chitosan nanoparticles was presented.

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