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Immunogenicity Evaluation of Recombinant *Staphylococcus aureus* Enterotoxin B (rSEB) and rSEB-loaded Chitosan Nanoparticles Following Nasal Administration

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ABSTRACT

Staphylococcal enterotoxin B (SEB), apotent superantigen, is responsible for many disorders caused by *Staphylococcus aureus*. With regard to the appearance of multidrug-resistant strains of the bacteria, there is a great need to develop an efficient vaccine against this pathogen. In the present study, the immunogenicity of recombinant SEB was evaluated following nasal administration to BABLB/c mice. Indeed, the rSEB protein was entrapped into chitosan nanoparticles and the immunogenicity of nano-formulation was investigated.

SEB protein was expressed in *E. coli* BL21 (DE3) and purified by using a nickel column. Chitosan nanoparticles were synthesized in the presence of rSEB; using the ionic gelation technique. Synthesized NPs containing rSEB and bare rSEB were administered to mice nasally.

Serum and stool IgG and IgA antibody showed that both formulations were able to evoke the mice's immune responses and there was no significant difference between them. Results of the toxin neutralization test on Vero cells indicated that the sera of the immunized mice had an inhibitory effect on the growth of these cells ($p<0.001$).

Nasal administration of bare rSEB could efficiently simulate the mice's immune system and nano-delivery of this protein via nasal route had not a significant impact on its immunogenicity improvement.

Keywords: Chitosan; Intranasal administration; Nanoparticles; *Staphylococcus aureus*; Staphylococcal enterotoxin B; Vaccines

INTRODUCTION

Staphylococcus aureus (*S. aureus*) is one of the most common causes of food poisoning all over the world.

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Staphylococcal food poisoning (SFP) is caused by the consumption of S. aureus enterotoxins.[1,2] Although it is a self-limiting disease, however, in some cases, it can lead to hospitalization, especially in young children or immunocompromised people.[3] Different enterotoxins and other related toxins have been identified which are produced by *S. aureus*. Pathogenic *S. aureus* isolates express at least one of these toxins.[4] One of the most import enterotoxins produced by this bacterium is

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enterotoxin type B (SEB), a superantigen with a molecular weight of 28 kDa and pI of about 8, which is very important due to its ability to be absorbed through inhalation and its bioterrorism potential applications as well. Structurally, it has 2 distinct domains with a very compact and complex tertiary structure. Due to the high compact structure, SEB is highly resistant to environmental conditions, especially gastrointestinal proteases, such as trypsin, papain and chymotrypsin.[2,5]

Because of the emergence of multi-drug resistant strains of *S. aureus*, which can be a crisis in medical environments,[6] vaccination is a proper strategy to combat the *S. aureus*-related diseases. There have been many efforts to develop an efficient vaccine against *S. aureus*, including recombinant subunit and whole-cell vaccines, which are well-reviewed in the literature.[7,8] Considering the role of SEB protein in the pathogenesis of *S. aureus*, it can be considered as a suitable candidate for vaccination against the agent.[9] There are many studies which have shown that there is a possibility of protection against SEB poisoning by passive immunization through administration of anti-SEB antibodies or active immunization through the administration of the protein through parenteral and oral routes.[9-14] However, there has not been any study on the immunogenicity of rSEB following the nasal administration.

Because of many advantages, especially, their safety, recombinant subunit vaccines are preferred over live-attenuated or inactivated vaccines. However, they are faced with many limitations. One of the limitations is their low efficiency, which makes it necessary to use appropriate adjuvants as well as delivery vehicles. Novel vaccine delivery systems, such as nanoparticles, can address two mentioned limitations: They can potentiate the host immune system and, at the same time, they can deliver the antigen to the immune system and they are widely used.[15-18] Despite the promising results in the improvement of the efficiency of many candidate vaccines, rare (if no) studies have evaluated the efficiency of NPs in the delivery of SEB. One of the mostly-used polymer materials in delivery nano-systems is chitosan. Due to its unique characteristics, such as non-toxicity, degradability, biocompatibility, and biodegradability chitosan is widely used in various fields.[19,20] Chitosan nanoparticles can protect their cargoes (biomaterials such as drugs, vaccines and nucleic acid fragments) from the harsh conditions of the mucosal route.[21] Indeed, because of invasiveness, mucosal delivery of the vaccines is preferred over parenteral administration of vaccines and vaccine candidates.

In the present study recombinant SEB was expressed in *E. coli* BL21 (DE3) cells and purified using a Ni-NTA column. The protein was entrapped into the chitosan NPs and the immunogenicity of bare rSEB and rSEB-loaded chitosan NPs were evaluated following the nasal administration to BALB/c mice. Indeed, two different concentrations of the protein were applied to investigate the effect of the administered dose in the elicitation of mice's immune responses.

MATERIALS AND METHODS

Ethics Statement

All animal experiments were performed according to the institutional guidelines of Animal Care and Use Sub-Committee at Imam Hossein University, Tehran, Iran, with the ethical code of 1821 dated back May-24-2017.

Reagent and Chemicals

Medium molecular weight chitosan (viscosity~200-800 cp), dimethyl sulfoxide (DMSO), 3-(4, 5-dimethyl-thiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT), trypsin–EDTA, penicillin, and tetracycline were obtained from Sigma–Aldrich (USA). Sodium tripolyphosphate (TPP), acetic acid, hydrochloric acid, ethanol (95%) and sodium chloride were prepared from Merck (Germany). The cell culture media (DMEM) and fetal calf serum (FCS) were purchased from Gibco (USA). All materials and reagents were of the HPLC grade.

Biological Molecules and Cell Line

In this experimental study, after bioinformatics analyzes, the *seb* gene was synthesized chemically by Biomatic Company (Canada), and inserted into the plasmid pET28a (+). The standard toxin was purchased from Sigma Aldrich Company. Secondary antibodies (conjugate) bound to HRP against mouse IgG and IgA and mouse antibodies against His-tag were purchased from Abcam Company (UK) and molecular markers from the Thermo Fischer Scientific (USA). The epithelial cell line of the monkey kidney (Vero) was prepared from the Iranian Biological Resource Center (IBRC).

SEB Expression and Purification

The sequence of *seb* gene was adopted from Gene Bank (Accession Number: M11118). After codon optimization, it was synthesized in pET28a (+). The recombinant construct was then transferred into *E. coli* BL21 (DE3) competent cells. Expression of the recombinant protein was induced by addition of isopropyl β- d-1-thiogalactopyranoside (IPTG) (final concentration of 1 mM). The expression of the desired protein was confirmed by Western blotting.[22]Since the recombinant protein had a histidine tag, the protein was purified by a Ni-NTA column (Qiagen). SDS-PAGE electrophoresis was used to evaluate the purified protein.

Preparation of Chitosan NPs Containing Recombinant SEB Protein

Ionic gelation method was used to prepare chitosan nanoparticles as previously described by Bagheripour et al[23] Sodium tripolyphosphate solution was prepared at a concentration of 1 mg/ml. To prepare a solution of chitosan with a concentration of 2 mg/mL, at first, 0.5 ml of glacial acetic acid 2% was added to 24.5 mL of distilled water. Then, 50 mL of chitosan powder was added to it. After one hour and complete dissolving of the chitosan powder, the pH of the solution was adjusted to 4.5 with 6N NaOH. 7.5 mL of the above solution was transferred to another container and 1 mg of protein was added dropwise to the chitosan solution. After 5 minutes, 5 mL of sodium tripolyphosphate solution was added dropwise at definitive intervals (60 minutes), and the solution was finally stirred for 30 minutes. Then, 100 µL of glycerol was poured into a falcon and the solution; containing the nanoparticles was transferred to a falcon and was centrifuged for 30 minutes at 21000g at 4°C. The pellet, containing chitosan nanoparticles, was stored and the supernatant was collected to investigate the loading efficacy of protein entrapment onto the nanoparticles.

Dynamic Light Scattering Study (DLS)

The DLS (Malvern Instruments, Malvern, UK) was used to investigate the hydrodynamic diameter, the dispersion index, and zeta potential of nanoparticles.

Investigation of Nanoparticle Morphology by Scanning Electron Microscopy (SEM)

To investigate the morphological characteristics of nanoparticles, a scanning electron microscope (model EM3200, KYKY Co) was exploited. In order to prevent the NPs' aggregation, freshly prepared specimens were placed on a glass surface. After complete drying, the sample was coated with a thin layer of gold by the physical vapor deposition (PV) method using Sputter Coater (KYKY SBC-12, Beijing, China). Finally, the sample was evaluated by the microscope. The size of NPs was analyzed by Image J software and the plot was drawn in SPSS software.

Investigation of the Particle Yield, Loading Efficiency, and Nanoparticle Loading Capacity

To determine the nanoparticle yield, after the preparation of nanoparticles, the nanoparticles were freeze-dried. Then, the weight of the dry matter was measured by a digital weighing scale and, according to the relation 1, the percentage of nanoparticle yield was calculated.

Particle Yield%=(Dried nanoparticle weight)/(Theoreticalmass (polymer+linker+protein))×100

Relation 1. Formula for calculating the percentage of nanoparticle yield

In this study, the indirect method was used to determine the loading efficiency (the amount of entrapped protein onto nanoparticles). To achieve this purpose, after the synthesis of SEB-loaded NPs, the suspension was centrifuged at 21000g for 20 minutes at 4°C. Then, the protein content of the supernatant was determined by the Bradford method. The percentage of loading efficiency and loading capacity of the nanoparticles was calculated using relations 2 and 3, respectively.

Loading Efficiency %=(Weight of loaded protein)/(Total weight of protein taken for loading)×100

Relation 2. The formula for calculating the percentage of loading efficiency

Loading Capacity %=(Weight of loaded protein)/(Weight of dried nanoparticle)×100

Relation 3. The formula for calculating the percentage of loading capacity

Investigation of the Stability and Durability of Recombinant SEB Protein during Preparation of Chitosan Nanoparticles

To investigate the possible effect of the chitosan nanoparticles production method on the stability and durability of recombinant SEB protein, after the optimization of the conditions of the nanoparticles production (low-speed stirring for 24 h at *p*H=4.5),

20 µL of the solution was electrophoresed on a 12% SDS-PAGE and the result was analyzed.

In Vitro Investigation of Antigen Release from Chitosan Nanoparticles

To determine the release profile of the protein from NPs, SEB-loaded chitosan NPs containing 1 mg protein was dissolved in 500 µL of Simulated Body Fluid (SBF)[24] and allowed to be agitated at 37°C and 200 rpm. At defined intervals (1, 2, 4, 6, 12, 24, 36, 48, 72, 96, 120, 144, 192 h) the mixture was centrifuged for 10 min at 21000 g. The supernatant was used for protein concentration determination (using Bradford assay) and 500 µL of fresh SBF buffer was added to the tubes. The test lasted for 8 days. Finally, the cumulative percentage curve of the released protein from the nanoparticles was plotted at each specified time.

In Vivo Studies of Nanoparticle on an Animal Model Determination of Serum and Fecal IgG and IgA

For the in vivo test, 48 female BALB/c mice (weighed 18-22 g) were grouped into six groups to evaluate the immunogenicity as well as the production of antibodies. Mice in groups 1 and 2 were intranasally administered with 10 and 20 µg of bare recombinant SEB protein, respectively. Mice of groups 3 and 4 received chitosan NPs containing 10 and 20 µg of recombinant SEB protein via nasal route, respectively. Groups 5 and 6 were the control groups that were administered with PBS and bare chitosan NPs through the nasal route. Administrations were carried out 4 times with 14-days intervals. In order to examine the humoral and mucosal immunity, IgG and IgA antibody titers were determined in serum and stool samples of animals. Blooding was performed 7 days after each administration and sera were collected and kept at -20°C. To prepare stool samples for IgA assay, 100 mg of stool was mixed with 300 µL of PBS. Then, the sample was centrifuged at 4000g for 25 min. After centrifugation, the supernatant was separated and 100µL of this solution was added to 10 µL PMSF (100 mM). The solution was then centrifuged at 21000g for 25 min and the supernatant was kept at -20°C. To measure the antibody titer in the sera and stool samples, the indirect enzyme-linked immunosorbent assay (ELISA) was used as described by Hajizade et al.[17] Here, 5 µg of rSEB was coated in the wells. For IgG assessment, anti-mouse IgG conjugated with HRP (Sigma, Germany) and for IgA assay, HRP conjugated anti-mouse IgA (Sigma, Germany) was used as the secondary antibodies.

In Vivo Challenge Test

For *in vivo* evaluation of the immunogenicity of each formulation, 75 days after the last administration, immunized and control mice were grouped into different groups for intraperitoneal and the oral challenge of SEB toxin. Before the challenge of the animals, LD50 of the SEB toxin (commercially prepared from Sigma Aldrich Co. (Germany)) as well as recombinant SEB was determined through Spearman-Karber method.[25]

Cell Culture Tests Evaluation of the Cytotoxicity of SEB Toxins (Recombinant and Standard SEB Proteins)

MTT standard test, with three replicates, was used to examine the toxicity effect of recombinant and standard SEB proteins on Vero cell line.[26] Concentrations of 1, 2, 5, 10 and 20 µg of recombinant and standard SEB proteins were used for the experiment.

Neutralization Assay of the Antisera of Immunized Animals

The neutralization power of the raised antibodies against SEB was evaluated through a neutralization test assay (NTA). To perform this assay, firstly, IC_{50} values for recombinant and standard SEB toxins were obtained. Then, serial dilutions of antisera were prepared (1/1, 1/10, 1/100, and 1/1000) and the preparations were added to 100 µg of SEB. The samples were incubated for 1 hour at 37°C. Finally, the mixture was added to the cell culture. The total volume of cells and solutions containing SEB proteins and anti-serum in each well were considered 100 µL.

Statistical Analyses

In order to test the normality of data, Shapiro-Wilk and Kolmogorov–Smirnov tests were used; using SPSS software (V.24). The comparison of the mean simple effects of administration amount, the administration group (different groups of mice) and the frequency of blood sampling and their interactions were measured by the Duncan test. The standard deviation of each of the parameters was also calculated. Probobility values less than 0.05 were considerd significant.

RESULTS

rSEB Expression and Purification

The sequence of *seb* gene before and after codon optimization can be seen in Figure 1. The codon adaptation index (CAI) of the optimized sequence changed from 0.68 to 0.82, which shows that the optimized sequence can be expressed in *E. coli* BL21 efficiently. Codon frequency distribution (CFD) analysis revealed that there is nor are codon in the optimized sequence. GC content of the sequence was changed from 22.24% before the optimization to 43.85% after the optimization.

Figure 2A shows the results of the protein expression. As can be seen in the figure, rSEB has been strongly expressed following the addition of IPTG. The protein yield was determined as 22 mg/L culture medium. We further determined the solubility of the expressed protein. Fig 2B shows that the protein is mainly expressed as soluble protein; therefore, the protein purification was performed; using a nickel column under non-denaturing conditions (Figure 2C). Finally, the expressed protein was confirmed by Western blotting (Figure 2D); using an anti-SEB antibody.

Investigating the Size Distribution and Zeta Potential of Nanoparticles by DLS

The results indicated that the protein-free nanoparticles had a size of about 25 nm and PDI=0.394 (Figure 3A), and nanoparticles containing protein of about 202 nm and PDI=0.590 (Figure 3B). The results in this section indicated that the size and PDI of the nanoparticles containing protein had been increased.

The results of zeta potential determination showed that protein-free chitosan nanoparticles had an average peak in the range of -16 mV, and chitosan nanoparticles containing protein also had an average peak of 10.6 mV (Figure 4).

Investigating the Morphology of Nanoparticles by Scanning Electron Microscopy (SEM)

The images of scanning electron microscopy showed that the synthesized NPs are finely spherical with a diameter of about 65 nm. The graph from the Image J software showed that the mean size distribution of the protein-free NPs by the electron microscopy was 25.41 nm (Figure 5).

Investigation of Particle Yield, Loading Efficiency, and Loading Capacity of Nanoparticles by the Indirect Method

In this part of the study, after calculating the particle yield, loading efficiency and loading capacity of nanoparticles containing SEB protein were determined by an indirect method, the results indicated that in all three parameters, the recombinant protein had the highest yields (Table 1).

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5'ATGGAAAATATGAAAGTTTTGTATGATGATAATC
ATGTATCAGCAATAAACGTTAAATCTATAGATCAAT
TTCTATACTTTGACTTAATATATTCTATTAAGGACA
CTAAGTTAGGGAATTATGATAATGTTCGAGTCGAAT
TTAAAAACAAAGATTTAGCTGATAAATACAAAGATA
AATACGTAGATGTGTTTGGAGCTAATTATTATTATC
AATGTTATTTTTCTAAAAAAACGAATGATATTAATT
CGCATCAAACTGACAAACGAAAAACTTGTATGTATG
GTGGTGTAACTGAGCATAATGGAAACCAATTAGATA
AATATAGAAGTATTACTGTTCGGGTATTTGAAGATG
GTAAAAATTTATTATCTTTTGACGTACAAACTAATA
AGAAAAAGGTGACTGCTCAAGAATTAGATTACCTAA
CTCGTCACTATTTGGTGAAAAATAAAAAACTCTATG
AATTTAACAACTCGCCTTATGAAACGGGATATATTA
AATTTATAGAAAATGAGAATAGCTTTTGGTATGACA
TGATGCCTGCACCAGGAGATAAATTTGACCAATCTA
AATATTTAATGATGTACAATGACAATAAAATGGTTG
ATTCTAAAGATGTGAAGATTGAAGTTTATCTTACGA
CAAAGAAAAAGTGA3'
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5'ATGGAAAACATGAAAGTTCTGTATGATGACAACC
ACGTTTCTGCGATCAACGTTAAATCTATTGACCAGT
TTCTGTACTTCGACCTGATCTACTCTATCAAAGACA
CCAAACTGGGTAACTACGACAACGTTCGTGTTGAGT
TTAAGAACAAAGACCTGGCGGACAAATACAAAGATA
AATACGTTGACGTGTTCGGTGCGAACTACTATTACC
AGTGCTACTTCTCTAAGAAAACCAACGACATCAACT
CCCACCAGACCGACAAACGTAAAACCTGCATGTACG
GTGGTGTTACCGAGCACAACGGCAACCAGCTGGATA
AGTATCGTTCTATCACCGTTCGCGTTTTCGAGGACG
GTAAGAACCTGCTGTCTTTCGACGTTCAGACCAACA
AGAAGAAGGTTACCGCGCAGGAACTGGACTATCTGA
CCCGTCACTACCTGGTTAAAAACAAAAAACTGTACG
AGTTCAACAACTCTCCGTACGAGACTGGCTACATCA
AATTCATTGAAAACGAAAACTCTTTCTGGTACGATA
TGATGCCGGCTCCGGGTGACAAATTCGATCAGAGCA
AATACCTGATGATGTACAACGATAACAAGATGGTTG
ACTCTAAAGACGTGAAAATCGAAGTTTACCTGACCA
CCAAAAAGAAATAA3'
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Figure 1. The sequence of *seb* gene before (left) and after (right) the codon optimization

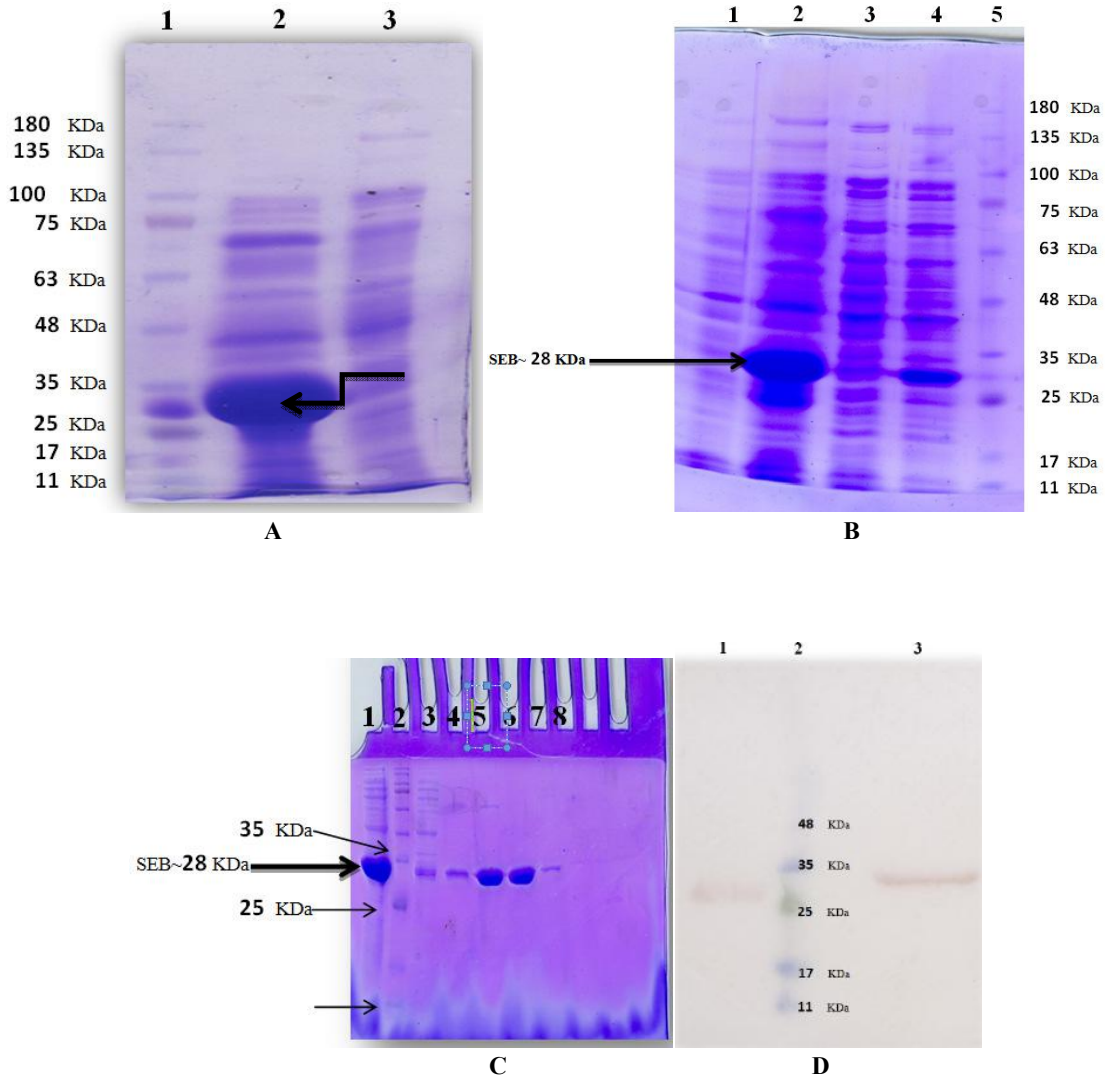
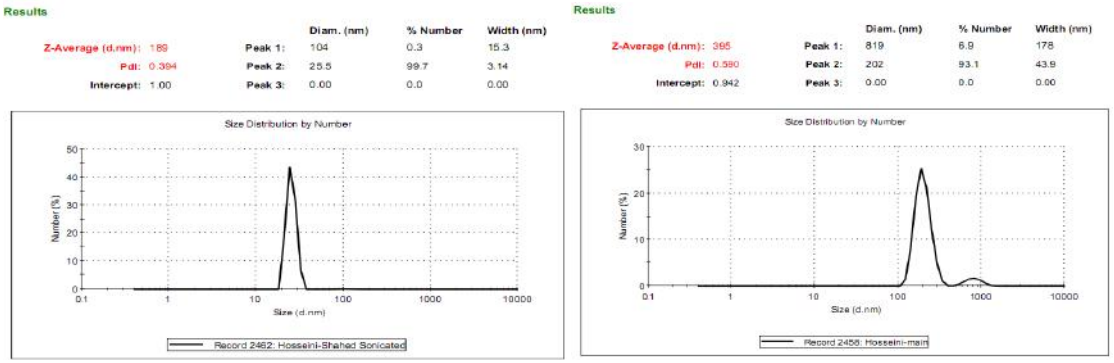


Figure 2. A: Expression of rSEB; Lane 1: Protein ladder; Lane 2: IPTG-induced sample; Lane 3: Uninduced sample. B: Determination of the protein solubility; Lane 1: Supernatant of the uninduced sample; Lane 2: Supernatant of IPTG-induced sample; Lane 3: Pellet of the uninduced sample; Lane 4: Pellet of the induced sample; Lane 5: Protein Ladder. C: rSEB purification with nickel column; lane 1: The sample before the application to the column; Lane 2: Protein Ladder; Lane 3: The flowthrough after the application of the sample to the column; Lane 4: The flowthrough after the addition of imidazole 20 mM to the column; Lane 5: The flowthrough after the addition of imidazole 40 mM to the column; Lane 6: The flowthrough after the addition of imidazole 100 mM to the column; Lane 7: The flowthrough after the addition of imidazole 250 mM to the column; Lane 8: The flowthrough after the addition of MES buffer to the column. D: Confirmation of the expressed protein using Western blotting; Lane 1: Commercially prepared SEB; Lane 2: Protein Ladder; Lane 3: rSEB. Different sizes of the two protein are due to the addition of a ≈4 kDatag to the recombinant protein



A B

Figure 3. Determination of chitosan nanoparticle size distribution by DLS: protein-free NPs (left) and NPs containing protein (right)

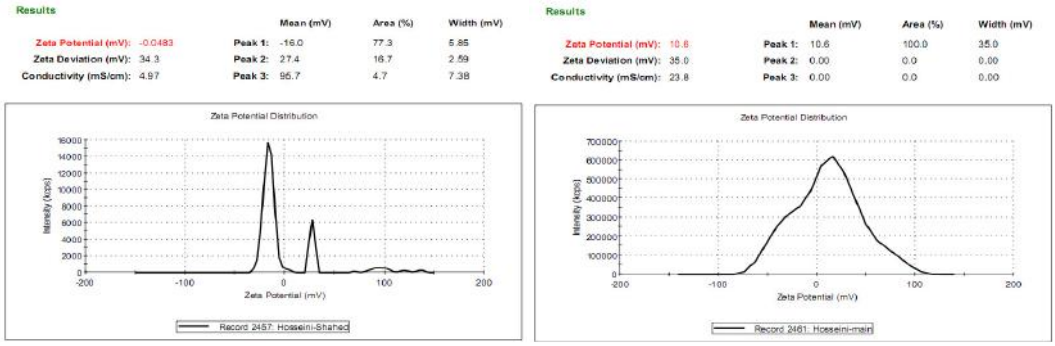
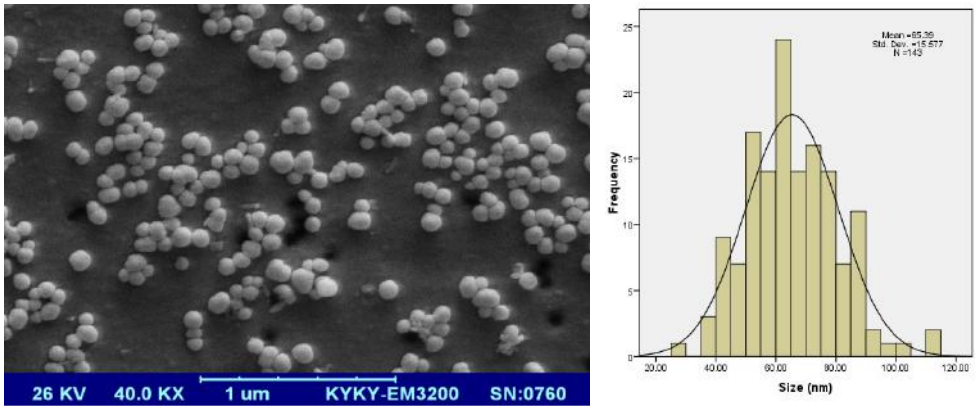


Figure 4. Determination of the zeta potential of chitosan nanoparticles in optimal conditions by DLS: protein-free NPs (left) and NPs containing protein (right).



A B

Figure 5. SEM image of SEB-loaded chitosan nanoparticles prepared by ionic gelation method (A) and chart of nanoparticle size distribution with ImageJ software (B)

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Table 1. Particle yield, loading efficiency and loading capacity of nanoparticles. Abbreviations: LC: Loading capacity; PY: Particle yield; LE: Loading efficiency

	LC%	PY%	LE%
Chitosan Nanoparticles containing SEB	5.72	76.19	89.7

Results of the Investigation of the Stability and Durability of Recombinant Protein during the Preparation of Chitosan Nanoparticles

Following the optimization of the nanoparticle production conditions, 20 µL of the solution was electrophoresed on a 12% SDS-PAGE. The results showed that the SEB protein during the synthesis of the chitosan nanoparticles was almost stable and a large amount of it had been loaded onto the synthesized nanoparticles (Figure 6).

In vitro Investigation of Antigen Release from Chitosan NPs

As shown in Figure 7, the process of protein release in the first 20 hours was fast, but after this time, the release process was approximately constant, and after eight days of release, about 50% of the protein was released from nanoparticles.

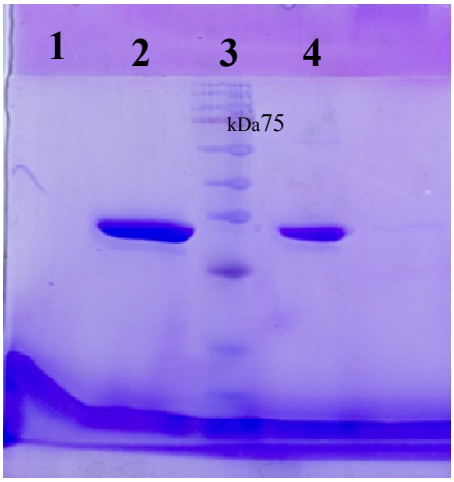

Figure 6. Result of the investigation of the stability and durability of recombinant protein during the preparation of chitosan nanoparticles on a 12% SDS-PAGE stained with Coomassie Blue. Lane 1: The recombinant protein as the control. Lane 2: Protein Molecular Marker. Lane 3: The chitosan nanoparticle-containing SEB protein (prepared at pH=4.5 with a low stirring rate) before the precipitation of NPs. Lane 4: The supernatant of the mixture after precipitation of chitosan nanoparticles

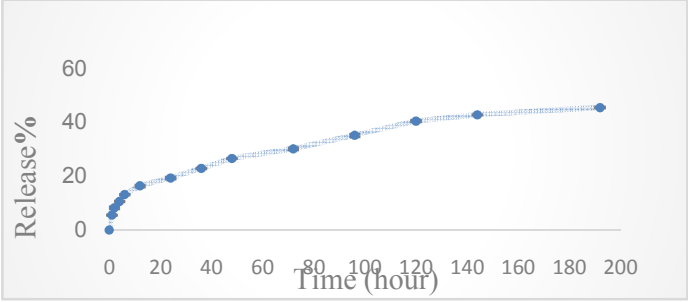

Figure 7. Results of cumulative accumulation of antigen release from chitosan nanoparticles for 8 days.

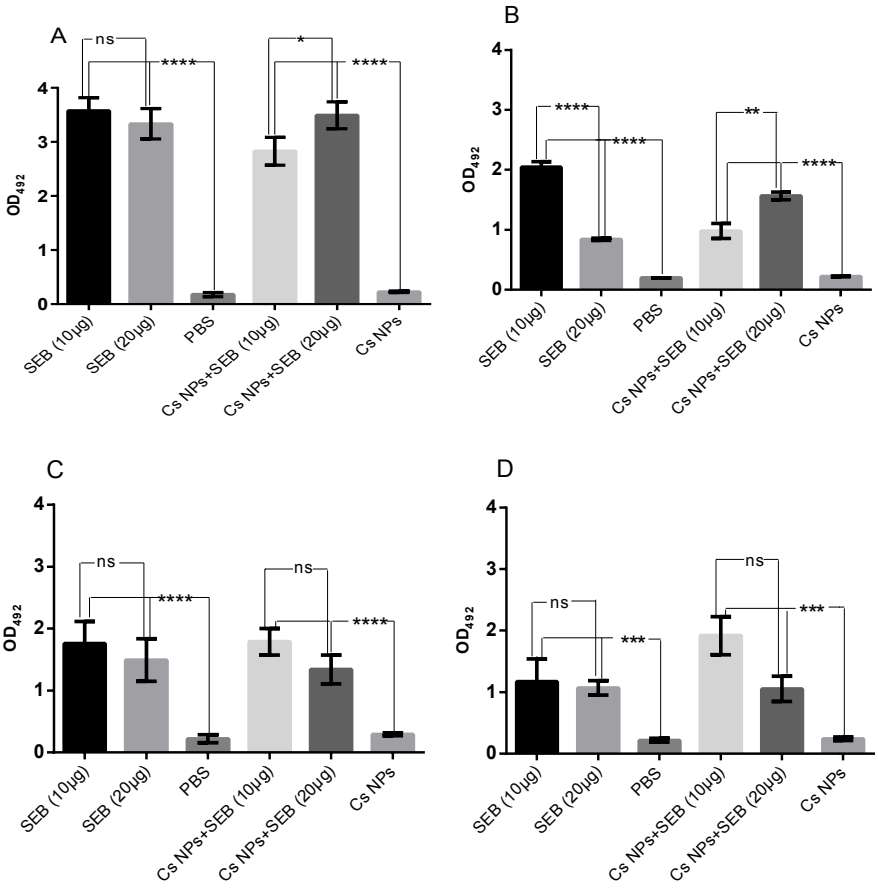

Figure 8. Investigation of IgG and IgA antibody titers in serum and stool specimens of the intranasally immunized and non-immunized mice; using ELISA. A: Serum IgG; B: Serum IgA; C: Stool IgG; D: Stool IgA. *: p<0.05; **: p<0.001; ***: p<0.0001; ****: p<0.00001; ns: not significant.

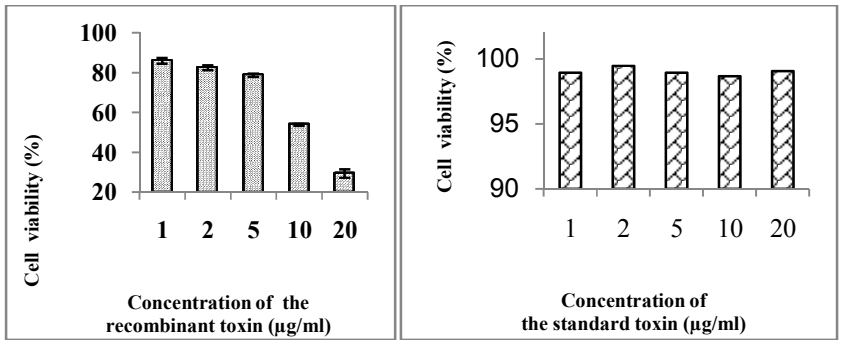

Figure 9. The percentage of Vero cells viability (epithelial cells of monkey kidney) with different concentrations of SEB recombinant protein (A) and standard toxin (B).

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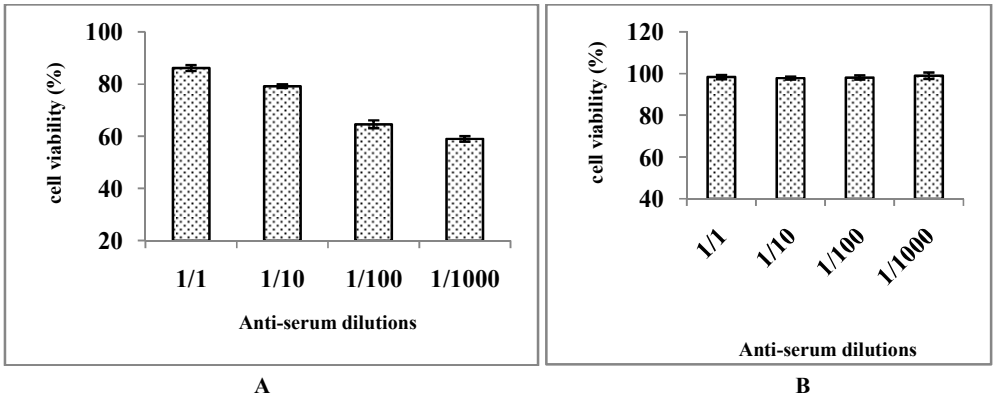

Figure 10. A: Vero cells' (epithelial cells of monkey kidney) viability in a challenge test with a constant concentration of recombinant toxin (100 µg/mL) and different concentrations of anti-serum dilutions; B: Vero cells' viability in the challenge test with a constant concentration of standard toxin (100 µg/mL) and different anti-serum dilutions

In Vivo Studies of Nanoparticles on Animal Model

After blood sampling and preparation of feces, serum IgG and IgA titers were analyzed by the ELISA method (Figure 8). As can be seen in Figure 8, the titers of serum IgG and IgA, as well as fecal IgG and IgA have raised in all test groups, while no raise in the titers of these antibodies in the sera and fecal specimens of the control groups was observed.

Cytotoxicity Assay

Evaluation of the Cytotoxicity of SEB Toxins (Recombinant and Standard SEB Proteins)

Results of MTT tests showed that recombinant SEB had a cytotoxic effect on the Vero cell line, while commercially prepared SEB toxin had not any cytotoxicity on this cell line (Figure 9). As can be seen in Figure 9, all applied concentrations of the recombinant SEB toxin (1, 2, 5, 10 and 20 µg) were cytotoxic for Vero cells. However, in none of the used concentrations of purchased SEB toxin (1, 2, 5, 10 and 20 µg), cytotoxicity was observed.

Neutralization Assay

Since none of the toxins had the ability to kill the animals, and it was not possible to conduct the animal challenge, a toxin neutralization assay was performed to assess the neutralization power of the raised antibodies in the sera of the immunized mice. All prepared dilutions of the sera (1/1, 1/10, 1/100, 1/1000) had a neutralization effect on SEB recombinant toxin. But, in the case of commercially prepared toxin, since the toxin was not toxic on the cells, not interpretable results were obtained. Figures 10A and 10B show the percentage of cell viability for recombinant and standard SEB proteins in the neutralization test, respectively.

DISCUSSION

Recombinant proteins are considered as safe and efficient vaccines. Staphylococcal enterotoxin B is a candidate vaccine which its immunogenicity has been proven in the parenteral route. Since the entry route of *Staphylococcus aureus* as well as enterotoxin B is mainly mucosal, mucosal immunization is so important in this regard. The mucosal delivery of antigens is faced with many limitations. Because of the presence of proteases and other conditions, the use of vaccine delivery systems has gathered many attentions in vaccine delivery.[27-29]

In this study, based on the advantages of nanoparticle systems in reducing the potential risks of SEB protein and its increased immunogenicity as novel vaccine delivery systems, a natural polymer-based nanoparticle (Chitosan) was used to deliver SEB recombinant protein, a potent immunogen of *S. aureus*[30] through nasal route. Furthermore, the immunogenicity of the bare protein through the nasal

route was also investigated. Two different concentrations of the protein (10 and 20 µg) were applied in both formulations, i.e., chitosan NPs and bare protein.

Investigation of the size and morphology of the synthesized NPs by DLS and SEM showed that the NPs had an average size of 202 and 65 nm, respectively. The observed difference in the measured size of the two techniques can be explained by the fact that in DLS, the hydrodynamic diameter of NPs is measured, which is greater than the actual size of the particles. This is an ideal size to pass through Peyer's patches and uptake by immune cells.[31] Indeed, it was shown that the protein is stable during the process of NPs synthesis. Analyzing the release of the protein from NPs showed that its release is controlled and sustained so that after 8 days about 50% of the protein did not release from the NPs. Investigation of the immunogenicity of NPs showed the efficiency of this formulation in evoking both IgG and IgA in serum and fecal specimens. According to the results of statistical analysis, both IgG and IgA titers in the serum were significantly higher in the group which received 20 µg of protein compared to those received 10 µg ($p<0.05$). However, both fecal IgG and IgA titers in the group which was administered with 10 µg of protein were significantly higher than those received 20 µg.

When the bare protein was nasally administered, serum IgG titer was not significantly different in the group which received 20 µg of the bare protein compared to those received 10 µg ($p>0.05$), while the titer of serum IgA in the group which received 10 µg of protein was significantly higher ($p<0.01$). However, the titers of fecal IgG and IgA were not significantly different in the two groups ($p>0.05$). Comparing the two formulations, i.e., protein-loaded NPs and bare protein showed no significant difference in fecal and serum IgG. However, the serum IgA titer in mice administered with 10 µg of the bare protein was significantly higher than those received protein-loaded NPs ($p<0.1$). The highest fecal IgA titer was detected in the group administered with NPs containing 10 µg of protein. The results of the present study are not compatible with the study of Bradley et al., where they found no SEB specific antibody following the nasal administration of bare SEB to mice.[32] However, there are studies that show nasal administration of bare antigens can evoke the immune responses, even more efficiently than adjuvant-formulated antigens. For example, in a research conducted by Bagheripour et al., the immunization of mice with the bare binding domain of botulinum neurotoxin type E (BoNT/E) and chitosan NPs containing BoNT/E showed that the bare antigen was more efficient than antigen-loaded NPs in stimulating the mice immune responses.[33]This phenomenon can be explained by the fact that there is low intra- and extracellular enzymatic degradation in the nasal route so those are antigens that can efficiently be taken by nasal immune cells. When antigens are loaded onto NPs, especially with techniques such as ionic gelation, in which antigens and polymer materials interact via ionic interactions and, as a result, the conformation of the antigens (determined mainly by ionic interactions) will be changed, their immunogenicity will be decreased.[34] However, Amini et al, reported that the recombinant ESAT-6 protein loaded onto chitosan NPs lead to an increase in its immunogenicity compared to the antigen alone.[35] Therefore, the results of this study and similar studies that used chitosan nanoparticles for nasal vaccine delivery indicated that the properties of the antigen-loaded onto chitosan nanoparticles have a great effect on the results of the immune system elicitation and the improvement or reduction of the antigen's immunogenicity. In the present study, two different concentrations of SEB protein (10 and 20 µg) were used to investigate the effect of SEB concentration on the efficiency of the immune responses and it was shown that the lower concentration (10 µg) can efficiently elicit the mice immune responses.

To evaluate the immunogenicity of a candidate vaccine, the animal challenge is a key strategy. However, because of the lack of an active SEB toxin, in the present study, we could not conduct this experiment. We applied even 300 times of LD_{50} of the toxin both through intraperitoneal and oral routes, but none of our control mice did not die. So, the neutralization effect of the sera of the immunized animals on the toxin was investigated in cell culture. According to the results of the toxicity test, the 100 µg/ml recombinant SEB (IC_{50}) can kill more than 50% of the cells in the cell culture. By keeping this amount constant for the recombinant and standard toxin and different dilution of the anti-serum after performing the MTT test, it was found that anti-serum had an inhibitory power of over 50% for recombinant toxin at all dilutions. However, in the case of the standard toxin, since this toxin had lost its toxicity, all cells survived

following the treatment with the toxin.

It did not escape from our notice that in bare SEB solution we had a minor LPS contamination; however, it was shown that the raised antibodies in the sera of the immunized mice were SEB-specific antibodies. Indeed, in the process of protein encapsulation, the majority part of LPS was eliminated and did not entrap in the NPs. Undoubtedly, the removal of LPS from the protein solution is so desirable; however, we have shown that the raised antibodies against rSEB were able to neutralize the toxin. In the case of commercially-prepared SEB, it is clear that the toxin had been inactivated (probably due to the inappropriate shipping conditions).

In conclusion, the results of the present study show that both bare recombinant SEB and chitosan NPs containing SEB can efficiently elicit the production of SEB-specific antibodies both in the sera and feces of Mice. These antibodies are efficient in the neutralization of the active SEB toxin. Furthermore, it was shown that the application of the lower concentration of SEB protein (10 µg/each administration) is more efficient in eliciting the immune responses.

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REFERENCES

1. Wakabayashi Y, Umeda K, et al. Staphylococcal food poisoning caused by Staphylococcus argenteus harboring staphylococcal enterotoxin genes. Int. J. Food Microbiol. 2018;265:23-9.
2. Rahimi F, Bouzari M, Katoli M, Pourshafi M. The prevalence Methicillin-resistant Staphylococcus aureus strains produce enterotoxin A in Tehran hospitals. Iran J Infect Dis Trop Med. 2014;19(65):59-68.
3. Murray R. Recognition and management of Staphylococcus aureus toxin-mediated disease. Intern. Med. J. 2005;35:S106-S19.
4. Thomas D, Chou S, Dauwalder O, Lina G. Diversity in Staphylococcus aureus enterotoxins. Superantigens and Superallergens. 93: Karger Publishers; 2007. p. 24-41.
5. Norouzi J, Goudarzi G, Pakzad P, Razavipour R. The isolation and detection of Staphylococcus aureus enterotoxins AE and TSST-1 genes from different sources by PCR method. Qom Univ. Med. Sci. J. 2012;6(3):78-85.
6. Hassoun A, Linden PK, Friedman B. Incidence, prevalence, and management of MRSA bacteremia across patient populations—a review of recent developments in MRSA management and treatment. Crit. Care Med.. 2017;21(1):211.
7. Ansari S, Jha RK, Mishra SK, Tiwari BR, Asaad AM. Recent advances in Staphylococcus aureus infection: focus on vaccine development. Infect. Drug Resist. 2019;12:1243.
8. Redi D, Raffaelli CS, Rossetti B, et al. Staphylococcus aureus vaccine preclinical and clinical development: current state of the art. New Microbiol. 2018;41:208-13.
9. LeClaire RD, Hunt RE, Bavari S. Protection against bacterial superantigen staphylococcal enterotoxin B by passive vaccination. Infect. Immun.. 2002;70(5):2278-81.
10. Michie CA. Staphylococcal vaccines. Trends Immunol. 2002;23(9):461-3.
11. Hale ML. Staphylococcal Enterotoxins, Staphylococcal Enterotoxin B and Bioterrorism. Bioterrorism: IntechOpen. 2012.
12. Karauzum H, Chen G, Abaandou L, Mahmoudieh M, Boroun AR, Shulenin S, et al. Synthetic human monoclonal antibodies toward staphylococcal enterotoxin B (SEB) protective against toxic shock syndrome.J. Biol. Chem. 2012;287(30):25203-15.
13. Coffman JD, Zhu J, Roach JM, Bavari S, Ulrich RG, Giardina SL. Production and purification of a recombinant staphylococcal enterotoxin B vaccine candidate expressed in Escherichia coli. Protein Expression Purif.. 2002;24(2):302-12.
14. Hudson LC, Seabolt BS, Odle J, Bost KL, Stahl CH, Piller KJ. Sublethal staphylococcal enterotoxin B challenge model in pigs to evaluate protection following immunization with a soybean-derived vaccine. Clin. Vaccine Immunol.. 2013;20(1):24-32.
15. Hajizade A, Salmanian AH, Amani J, Ebrahimi F, Arpanaei A. EspA-loaded mesoporous silica nanoparticles can efficiently protect animal model against enterohaemorrhagic *E. coli* O157: H7. Artif. Cells, Nanomed., Biotechnol. 2018:1-9.
16. Kordbacheh E, Nazarian S, Hajizadeh A, Sadeghi D. Entrapment of LTB protein in alginate nanoparticles protects against Enterotoxigenic Escherichia coli. Apmis. 2018;126(4):320-8.
17. Kordbacheh E, Nazarian S, Sadeghi D, Hajizadeh A. An LTB-entrapped protein in PLGA nanoparticles preserves against enterotoxin of enterotoxigenic Escherichia coli.

Iran. J. Basic Med. Sci. 2018;21(5):517.
18. Gause KT, Wheatley AK, Cui J, Yan Y, Kent SJ, Caruso F. Immunological principles guiding the rational design of particles for vaccine delivery. ACS nano. 2017;11(1):54-68.
19. Zhao L, Seth A, Wibowo N, Zhao C-X, Mitter N, Yu C, et al. Nanoparticle vaccines. Vaccine. 2014;32(3):327-37.
20. Mengelizadeh N, Haghighifard NJ, Takdastan A, Hormozinejad M. Physicochemical Characterization of Biopolymer Chitosan Extracted from Shrimp Shells. Sci. Technol.. 2015;27(6):371-80.
21. Kumar A, Vimal A, Kumar A. Why Chitosan? From properties to perspective of mucosal drug delivery. Int. J. Biol. Macromol. 2016;91:615-22.
22. Hosseini SA, Ebrahimi F, Nazarian Sh, Hamidi M. Recombinant Expression of *Staphylococcus Aureus* Enterotoxin Type B as a Vaccine Candidate. Jundishapur Sci Med J. 2017; 16(6):653-664.
23. Bagheripour MJ, Ebrahimi F, Hajizade A, Nazarian S. Immunogenicity evaluation of rBoNT/E nanovaccine after mucosal administration. Iran. J. Basic Med. Sci.2019;22(4):353.
24. Marques MR, Loebenberg R, Almukainzi M. Simulated biological fluids with possible application in dissolution testing.Dissolution Technol. 2011;18(3):15-28.
25. Ramakrishnan MA. Determination of 50% endpoint titer using a simple formula. J. Virol. 2016;5(2):85.
26. Hayon T, Dvilansky A, Shpilberg O, Nathan I. Appraisal of the MTT-based assay as a useful tool for predicting drug chemosensitivity in leukemia. Leuk. Lymphoma 2003;44(11):1957-62.
27. Hajizade A, Ebrahimi F, Salmanian A-H, Arpanae A, Amani J. Nanoparticles in vaccine development. J. Appl. Biotechnol. Rep. 2015;1(4):pp. 125-34.
28. Fini A, Orienti I. The role of chitosan in drug delivery. Am. J. Drug Delivery 2003;1(1):43-59.
29. Bagheripour M, Ebrahimi F, Hajizadeh A, Nazarian S. Immunogenicity Effect of Chitosan Nanoparticles Containing Botulinum Neurotoxin E binding Domain Recombinant Protein in Mice. J. Mazandaran Univ. Med. Sci. 2015;25(124):37-47.
30. Buelow R, O'Hehir R, Schreifels R, Kummerehl T, Riley G, Lamb J. Localization of the immunologic activity in the superantigen Staphylococcal enterotoxin B using truncated recombinant fusion proteins. J. Immunol. 1992;148(1):1-6.
31. Shang L, Nienhaus K, Nienhaus GU. Engineered nanoparticles interacting with cells: size matters. J. Nanobiotechnol. 2014;12(1):5.
32. Stiles BG, Garza AR, Ulrich RG, Boles JW. Mucosal vaccination with recombinantly attenuated staphylococcal enterotoxin B and protection in a murine model. Infect. Immun. 2001;69(4):2031-6.
33. Bagheripour M, Ebrahimi F, Hajizadeh A, Nazarian S, Arefpour M. Preparation of chitosan-based botulinum neurotoxin e recombinant nanovaccine and evaluation of its immunogenicity as oral & intradermal route in mice. J RafsanjanUniv Med Sci 2016;14(11):923-38.
34. Fernández-Urrusuno R, Calvo P, Remuñán-López C, Vila-Jato JL, Alonso MJ. Enhancement of nasal absorption of insulin using chitosan nanoparticles. Pharm. Res. 1999;16(10):1576-81.
35. Amini Y, Tebianian M, Mosavari N, Fasihi Ramandi M, Ebrahimi S, Dabaghian M, et al. Preparation of ESAT-6 Nanoparticles and Evaluation of Humoral Immunity after Intranasal Administration. JSSU. 2013;20(5):612-26.