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Original Article

Design and Development of a New Method for the Production of Nanotoxoids from *Clostridium Perfringens* Beta Toxin

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Abstract

In recent years, a nanoparticle-based strategy has shown that non-denatured protein toxins can be used to enhance the appropriate immune response. Once the toxin reacts between the nanoparticles and the protein (toxin), it loses its toxicity because it does not attach to its ligand at the cell surface. The results of the nanoparticle and toxin complex show that the nanoparticles facilitate the internal release of the toxin. *Clostridium perfringens* beta toxin is produced by *Clostridium perfringens* type B and C, and diarrhea is the most important disease caused in newborn lambs. When beta toxin forms a complex with nanoparticles, the reaction between the toxin and the nanoparticle leads to the formation of a new form of nanoparticle in which the toxin loses its lethality due to its involvement; therefore, it becomes a toxoid. The nanoparticles used in this research are of poly lactic-co-glycolic acid (PLGA) type, one of the most developed biodegradable polymers. This study aimed to isolate and purify *Clostridium perfringens* beta toxin and produce its complex with PLGA nanoparticles to form a non-toxic structure. In this study, *Clostridium perfringens* beta toxin type B was isolated using ammonium sulfate precipitation and gel filtration chromatography. Toxin assay was performed *in vivo* (lethal dose [LD50]) and *in vitro* by sodium dodecyl sulphate-polyacrylamide gel electrophoresis at each stage, and the quantity of purified toxin was calculated to be 10 mg/ml. Afterward, the beta toxin antigen was used as the basis for the preparation of nanotoxoid candidates with nanoparticle formulation. Moreover, the PLGA polymer and water-oil-water methods were used to fabricate nanoparticles. Under optimal conditions, nanoparticles without antigen with an average size of 100 nm and zeta potential of -23.28 mV, as well as nanoparticles containing antigen with an average size of 120 nm and zeta potential of -18.2 mV, were prepared. When nanoparticles are injected into mice with the beta toxin, the toxin becomes a toxoid with no toxicity effects, and it cannot bind to its receptors and reveal its effects. In this study, the mice showed mild symptoms in one case, and none of them died. The beta and PLGA toxin model could also be applied as a candidate to study the release and immunization of the target animal. In order to achieve antigen regulation using natural polymers, it is recommended to conduct a comparative study between nanoparticles based on natural polymers.

Keywords: Beta toxin, Clostridium perfringens, Nanoparticles, Poly lactic-co-glycolic acid

1. Introduction

Clostridium perfringens is a Gram-positive, anaerobic, and encapsulated bacillus, which mainly causes enterotoxemia in different hosts. Toxins produced by *C. perfringens* play an important role in its pathogenicity. Among all of these toxins, the beta toxin

is the most important which is produced by types *B* and *C* and is sensitive to proteolytic enzymes (1). This toxin is a beta pore-forming toxin (B-PFT) that has a homologous structure and changes naturally in the presence of intestinal enzymes and increases the permeability of intestinal capillaries (2), which causes bleeding areas and mucosal ulcers in the small intestine. The most important disease caused by *C. perfringens* type *B* is bloody diarrhea for newborn lambs. The *Cpb* gene in type *B* is a plasmid with a molecular weight of 90 kDa responsible for the production of toxin *B* (3). The beta toxin can be used to design and manufacture calf diarrhea vaccines and be purified as much as possible. The most important issue after purifying a toxin is converting it to a toxoid so that the toxin's immunogenicity, potency, and nature are not affected. For this purpose, various methods for producing toxoids from toxins have been developed, one of which is the use of biological polymers and the production of nanoparticles containing toxins. Encapsulation of toxins in nanoparticles is an excellent way to present beta toxin to immune cells, which are accompanied by slow toxin release (4). In order to combine the beta toxin with the nanoparticles, the toxin must be concentrated and purified as much as possible. As of 1975, several studies have been performed on *C. perfringens* beta toxin type *C*, and all the studies used ammonium sulfate to concentrate the toxin, and the recovery from 24% to 40% was determined. In these studies, the isoelectric point 5.6 was also determined. In 2014, in the department of Anaerobic Bacterial Vaccine, Research and Production in Razi Institute, Iran, ammonium sulfate and chromatographic column were used to concentrate and purify toxin beta type *C*. However, beta toxin type B has not been purified and combined with nanoparticles so far to be regarded as a nanotoxoid. Recent efforts in the development of toxoids intend to preserve the native virulence and structure of the toxin, thereby improving both its potency and immunogenicity (4). However, one of the proposed solutions for the formulation and delivery of vaccines containing the beta toxin is its encapsulation in nanoparticles to increase its release time in the body and the duration of its exposure to the immune system. Some proteins, such as beta-toxins, have unique anti-phagocyte properties and long shelf life when combined with nanoparticles and placed on the wall of cells, such as erythrocytes (5). Recently, biodegradable nanoparticles, such as poly lactic-co-glycolic acid (PLGA), have been placed in the membrane of cells, such as RBCs, to be secretly camouflaged in the immune system and studied for therapeutic purposes (6). The PLGA is one of the most developed biodegradable polymers and is extensively used for therapeutic purposes because of its attractive properties. Due to hydrolysis, PLGA is degraded by contact and presence in water in erosion bulk, as well as lactic acid, and glycolic acid monomers are produced. These monomers are the products of different metabolic pathways of the body, such as the Krebs cycle, and are eliminated from the body by normal metabolic pathways (7). This study aimed to isolate and purify beta toxin from *C. perfringens* type *B* and produce its complex with PLGA nanoparticles as a nanotoxoid to form a non-toxic structure that does not cause local effects at the injection site. These nanoparticles can be studied in the future with a biological carrier in a living organism, such as red blood cells (8).

2. Materials and Methods

2.1. Bacterial Culture and Toxin Harvesting

C. perfringens type *B* is a strain that produces beta toxin; however it does not produce epsilon toxin (9). It was cultured for 6 hours and evaluated in supernatant for minimum lethal dose (MLD), bacterial purity, and protein content. In addition, one liter of bacterial culture was centrifuged (International centrifuge Model FS) for 45 min at 5000 rpm, and the supernatant was prepared to add ammonium sulfate (10).

2.2. Ammonium Sulfate Precipitation

In order to determine the appropriate amount of ammonium sulfate (Scharlau Co.), different concentrations were added to the supernatant, and finally, it was figured out that the best concentration of

ammonium sulfate is 50% added in two steps. Firstly, ammonium sulfate (25%) was added to the supernatant on the stirrer, which was completely dissolved from 1 to 2 h and incubated for 4 h at 4°C. The suspension was then centrifuged at 5000 rpm for 45 min, the second stage of ammonium sulfate (25%) was added to the supernatant and incubated again for 24 h at 24°C on a magnetic stirrer to complete the dissolution (11).

2.3. Isolation of Toxin

The prepared suspension was centrifuged for 45 min at 5000 rpm, and the precipitate was washed with 20 ml of tris-hydrochloride buffer solution at a concentration of 20 mM and was ready for dialysis against 20 mM of the tris-hydrochloride buffer. The duration of dialysis was 48 h, and the dialysis buffer was changed twice. Afterward, the solution was centrifuged at 8000 rpm for 45 min, and the supernatant was clarified by filter (0.45 micron) to make the solution clear (12).

2.4. Chromatography

Separation of proteins was performed based on gel filtration chromatography (the column packed in Razi Institute, Iran). For this purpose, the gel was washed with tris-hydrochloride buffer solution, 20 ml of the sample was loaded on the column, the amount of output was adjusted to 20 drops per minute, and the output fractions in the test tubes in a volume of 2.5 ml were collected and evaluated (13). The optical density of samples was read at 280 nm (Ut Trospec 2000, Pharmacia Biotech), the protein concentration was calculated using a standard curve, and the protein pattern was determined using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE).

2.5. Measurement of the Minimum Lethal Dose

Dilutions of 1.100 to 1.1500 were prepared from each fraction, and 0.5 ml was intravenously injected into two NIH mice weighing 16 to 18 g, and the mortality of mice was evaluated within four days.

2.6. Elimination of Possible Proteins in the Collected Sample

In order to evaluate pure beta protein, alpha and epsilon proteins should be removed by the probability intervention. An amount of 0.5 ml of prepared solution was mixed with 0.1 ml of alpha-toxin (lyophilized in the Department of Anaerobic Bacterial Vaccine Research and Production in Razi Institute, Iran), 0.2 ml of normal saline was injected intracutaneously to a guinea pig, and the cutaneous reaction was evaluated.

2.7. Western Blot Analysis

The presence of beta toxin in the purified sample was confirmed by Western blotting using Sheep Anti-beta monoclonal antibody (120 IU/ml) (Department of Anaerobic Bacterial Vaccine Research and Production, Razi Institute, Iran).

2.8. Preparation of PLGA Nanoparticles and Nanotoxoid

The PLGA (Sigma) nanoparticles containing beta toxin of *C. perfringens* were synthesized by the water-oil-water double emulsion solvent evaporation method. First, the organic phase of 1% PLGA in acetone was prepared, and afterward, the inner aqueous phase containing beta toxin ($\frac{\text{Betatoxin}}{\text{PLGA}}$=0.1) was emulsified in organic PLGA solution under stirrer (VELP, Italy) condition, and at the next step, it was sonicated for 1 min (Elma, Germany). After preparing the first emulsion, it was emulsified in an external aqueous phase (w2) containing 1.25% poly-vinyl alcohol (PVA) in a volume 2.5 times that of the entire first emulsion and then sonicated for 1 min. After ultrasonic treatment, the acetone was evaporated overnight under a magnetic stirrer at 800 rpm. The next day, the particles were collected after centrifugation (HERMLE, Germany) for 25 min at 14000 rpm, and they were washed two times by water to remove encapsulated toxin and wash excess PVA. The formed nanoparticles were characterized by DLS (Maven, Denmark) with an average particle size of ≤100 nm. The average particle size was measured using a DLS device, and due to the optimal particle size and appropriate weight range, the desired protein was added to it and re-evaluated "toxin+nanoparticles" (14).

2.9. Evaluation of Nanotoxoid Residual Toxicity

In order to investigate the antigenicity change in the form of toxin and nanoparticle binding, *in vivo*

experiments were performed on mice. The weight of the NIH mice was 16-18 g, and there were 10 mice in each group. In order to evaluate the local and general effects of mice, three subcutaneous injections were performed on days 0, 14, and 28. After the formulation, 0.5 ml of each compound was injected into one mouse. The experimental groups were as follows:

1- Physiological serum (control group)
2- PLGA nanoparticles
3- Beta toxin
4- Nanoparticles with antigen: for the formulation of this group according to the amount of lethal dose (LD50) and the amount of protein, the ratio between them=0.1 protein/PLGA.

The concentration of protein (toxin) was equal to 10 mg/ml.

3. Results

3.1. Purification of *Clostridium Perfringens* Beta Toxin Type B

The crude toxin protein (toxin-containing culture medium) and purified toxin were evaluated and then compared for MLD per unit volume and SDS-PAGE analysis.

The result of table 1 showed that the amount of nonspecific proteins decreased with increasing toxicity, and the results also showed that the efficiency of the purified toxin was eventually in the range from 40% to 50%.

As indicated in figure 1, the electrophoresis of the samples extracted from the chromatographic column showed pure bands in fractions from 6 to 10 on 3% polyacrylamide gel. The molecular weight of beta toxin was identified to be approximately 40 kDa. In addition, the purified proteins were significantly reacted with anti-beta toxin antibody in the western blot analysis, indicating the protein's high purity. Moreover, observations from intradermal injection into piglets showed no skin symptoms, such as skin discoloration (purple) at the injection site.

3.2. Western Blots Results and Neutralization of Interfering Toxins

The western blotting test (figure 2), was used to detect specific proteins in the samples obtained from SDS-PAGE. The use of 1.100 solution of immunoglobulin G conjugate antibody indicated that the proper binding of antibodies and antigen (brown) had been performed in this test.

Table 1. Results of toxicity rate, crude protein rate, and percentage of product recovery

No. Sample	Crud Toxin		Purified Toxin		Recovery %	
	Toxicity (MLD)	Amount of Toxin	Toxicity (MLD)	Amount of Toxin	Crud Toxin	Purified Toxin
1	1.100	9.3 mg/ml	1.800	2.3 mg/ml	------	40%
2	1.100	10.3 mg/ml	1.800	2.5 mg/ml	------	40%
3	1.100	10.9 mg/ml	1.1000	1.83 mg/ml	------	50%

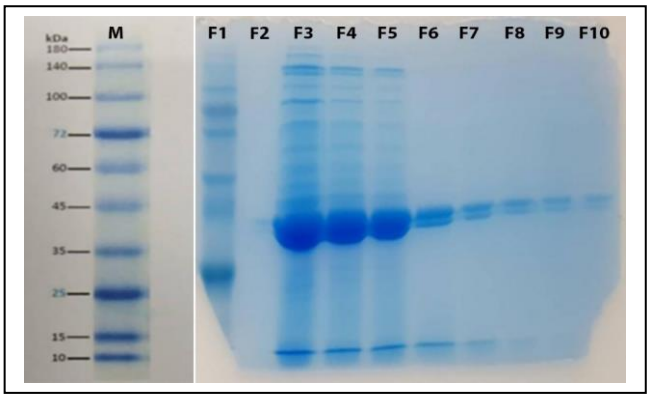

Figure 1. SDS-PAGE of the collected fractions (F1 to F10) from the chromatographic column and the corresponding ladder

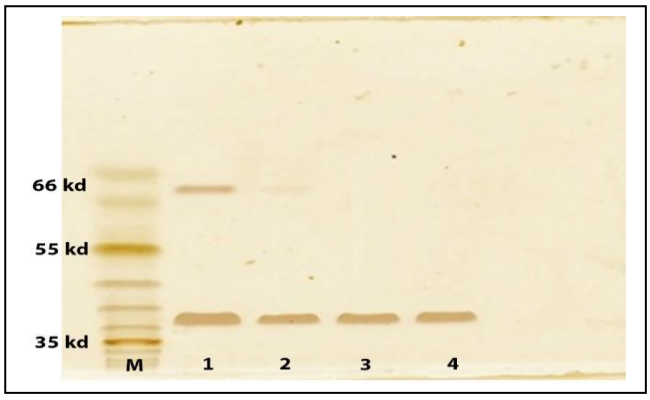

Figure 2. Western blot results; L line indicates the molecular weight of the respective ladder, and samples (2 to 4) indicate the specificity of the purified beta proteina

3.3. Evaluation of Nanoparticles

3.3.1. Size Distribution and Zeta Potential of Antigen-Free PLGA Nanoparticles

Figure 3 shows the nanoparticle size distribution based on intensity. Examination of particles by DLS device indicates that antigen-free nanoparticles with an average particle size of 100 nm and polydispersity index (PDI) of 0.082 are formed. Moreover, zeta potential antigen-free nanoparticles were measured at -23.8 mV.

3.3.2. Size Distribution and Zeta Potential of Toxin-Containing Nanoparticles

Figures 4 and 5 indicate the particle size distribution based on intensity. The measurement of antigen-containing nanoparticles by DLS showed that the particles were formed with an average size of 100 nm and a PDI of 0.357.

In figure 6 shown that nanoparticles containing antigen with an average size of 120 nm and zeta potential of -18.2 mV.

3.4. Evaluation of Nanotoxoid Antigenicity

As indicated in table 2, almost all of the mice receiving the toxin had diseases and ulcers at the injection site, whereas the mice that only received the PLGA nanoparticle did not have a health-related complication at the injection site. In addition, only one mouse showed mild signs of the disease. Moreover, none of the mice, which received the toxin with PLGA, died which suggests that when nanoparticles were injected into mice with the beta toxin, the toxin was converted to a toxoid that had no toxicity effects, and the toxin was not delivered to the recipient.

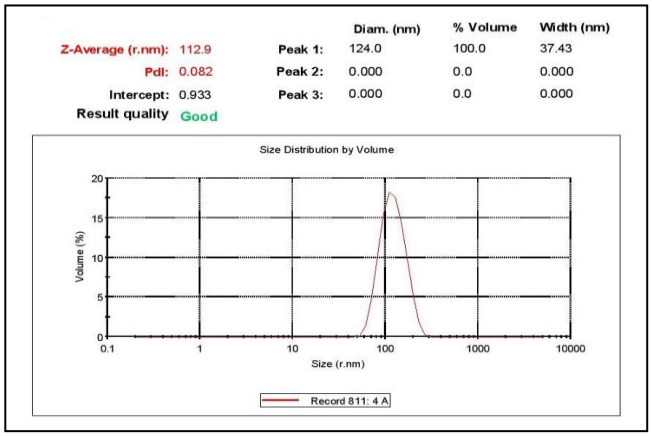

Figure 3. Antigen-free size distribution of PLGA nanoparticles

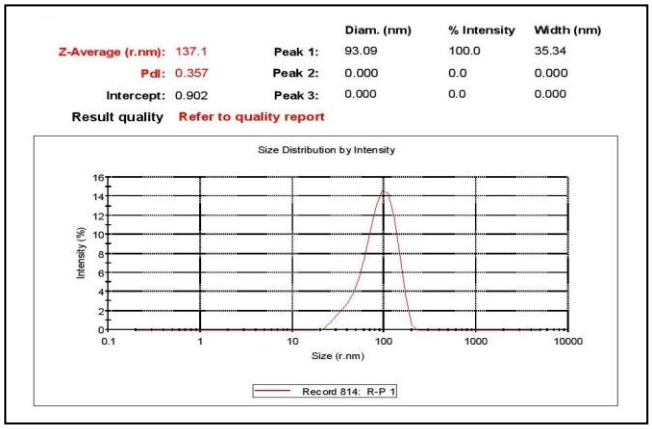

Figure 5. Size distribution of PLGA nanoparticles containing *Clostridium perfringens* beta toxin

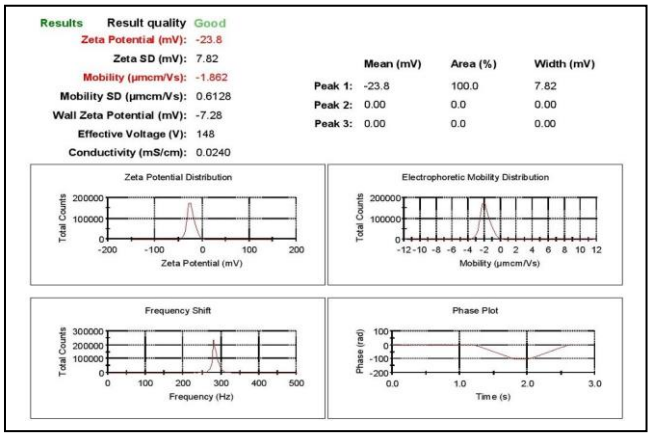

Figure 4. Antigen-free zeta potential of PLGA nanoparticles

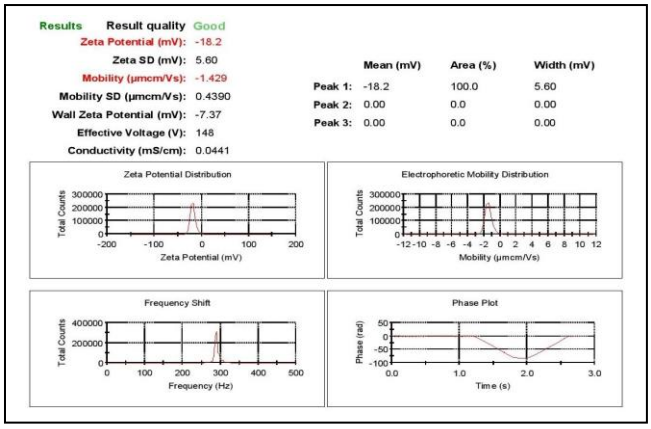

Figure 6. Zeta potential of PLGA nanoparticles containing *Clostridium perfringens* beta toxin

Table 2. Results of *in vivo* experiments on laboratory mice

Sample	Amount of Injection	Route of Injection	No. of Mice	No. of Healthy Mice	No. of Dead Mice	Percentage of Healthy Mice
Normal Saline	0.5 ml	ID	10	10	0	100%
PLGA Nanoparticle	0.5 ml	ID	10	10	0	100%
Beta Toxin	0.5 ml	ID	10	0	10	0%
PLGA+Beta Toxin	0.5 ml	ID	10	9	1* 0 †	90%

*Sick mouse refers to a mouse with complications, such as lethargy, abdominal indentation, and ulcers at the injection site
† Dead mouse refers to a mouse that is dead at this time

4. Discussion

C. perfringens toxin type *B* is the second toxin of *C. perfringens*, the LD50 of which is approximately equal to 400 ng/kg body weight of mice. The molecular weight of beta toxin is 35 kDa, and it is a B-PFT with a homologous structure (15). The acute form of this toxin can bind to vascular endothelial cells in intestinal tissue and cause thrombosis, resulting in intestinal necrosis and enteritis (16).

The researchers isolated 19 strains of *C. perfringens* type *B* with or without trypsin from the gastrointestinal tract and injected them intravenously by the MLD method, which showed a correlation between LD50 and *Cpb* levels in *C. perfringens* toxin type *B* culture. They showed that when samples were mixed with α monoclonal antibody and injected, no complications were observed in guinea pigs (intradermal). In addition, neutralization with a beta monoclonal antibody against *Cpb* reduces mortality by the injection of supernatant culture (15).

Recent advances in biology and materials have led to much interest in proteins that form nano-complex particles with unique anti-phagocyte properties and longer shelf life. Some proteins, such as beta toxins, when combined with nanoparticles, can be placed on the walls of red blood cells to have unique anti-phagocyte properties and longer shelf life in the body. In 2019, Zia et al. declared that beta toxins were associated with proteins (toxins) when they were in the membrane process, such as red blood cells due to camouflage in the body's lymphatic system and can be studied for therapeutic purposes (17).

The PLGA is one of the most developed biodegradable polymers, which is frequently used in drug delivery purposes due to its separating properties and the fact that its hydrolysis produces metabolic monomers. Lactic acid and glycolic acid are easily metabolized and cleared by the body through the Krebs cycle. The use of PLGA in human drug delivery systems is approved by the Food and Drug Administration and the European Medical Agency (18). The formulation of nanoparticles depends on the appropriate polymer system that has the greatest potential for encapsulation, promotion of biodegradability, and shelf life. The choice of nanoparticles depends on the sizes, surface charges, and hydrophobicity of nanoparticles. The size, tissue and cell biocompatibility and nontoxicity of a nanoparticle carrier for transportation of a drug, protein, peptide, and nucleic acid molecules through the cell membranes and the size of targeted cell dimensions are important factors which are required to be considered for a successful transportation (19).

In the present study, the initial design of an antigen delivery system was performed using PLGA nanoparticles, and while examining the purity of antigen (toxin type *B*), the role of PLGA polymer as a biodegradable and biocompatible polymer was evaluated. In the first separation stage, the purification of *C. perfringens* toxin type *B* from vaccine strain was discussed, which is routinely used to make enterotoxaemia vaccine. Isolation and purification methods were studied, and the best approach was selected (20) using centrifugation and suitable

ammonium sulfate (50%). The dialysis and salt separation and then chromatography column were obtained using G50 column of pure toxin with high qualitative and quantitative efficiency percentage. Afterward, by confirming pure toxin in terms of MLD, SDS-PAGE, and neutralization of the toxin with type B, the monoclonal antibody started to make suitable PLGA nanoparticles in terms of size (approximately 100 nm, as well as suitable zeta potential and PDI). In this study, many advantages of PLGA nanoparticles have been considered relying on some studies indicating their desirable properties for the preparation of nanoparticles (21). The preparation method was water-oil-water emulsion (w-o-w), and the optimal conditions of nanoparticles were obtained by successive measurements of their physicochemical properties (22).

The size and diffusion of PLGA nanoparticles coated with the toxin (protein) were investigated using DLS. It was observed that the appropriate concentration of the polymer is equal to 0.1%, and the particles are within the acceptable size range of 100 nm. The significant protein release from PLGA nanoparticles indicated a balanced release process. The injection of PLGA+toxin complex indicated that this complex was asymptomatic in terms of appearance at the injection site, and the animal showed this symptom after receiving the solution. There was no lethargy or disease signifying that this complex was the cause of them, which was due to the spatial deformation of the toxin. This substance cannot bind to its specific receptors and has acted as a toxoid that can be determined by further investigation of its immunogenicity. The PLGA+beta toxin model could be a candidate for animal release and immunization. Additional studies, such as the transport of nanoparticles with the toxin by red blood cells, have been suggested by some researchers studied on some toxins, such as *Staphylococci*, to achieve antigen systems using natural polymers. Finally, it is recommended to conduct a comparative study between nanoparticles based on natural polymers.

Authors' Contribution

Study concept and design: E. A.
Acquisition of data: E. A., M. T., T. Z. S., M.T., and R. Y.
Analysis and interpretation of data: M. T. and E. A.
Drafting of the manuscript: R. T., T. Z. S., and E. A.
Administrative, technical, and materials support: T. Z. S. and R. T.

Ethics

All the ethical standards were approved by the ethics committee of the Razi Vaccine and Serum Research Institute, Karaj, Iran.

Conflict of Interest

The authors declare that they have no conflict of interest.

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