Iranian Journal of Basic Medical Sciences
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IJBMS

# An LTB-entrapped protein in PLGA nanoparticles preserves against enterotoxin of enterotoxigenic *Escherichia coli*

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**ARTICLE INFO**

***Article type:***
Original article

***Article history:***
Received: Oct 19, 2017
Accepted: Sep 28, 2017

***Keywords:***
Enterotoxigenic *Escherichia coli*
Heat-labile enterotoxin
Immunization
Nanoparticle
PLGA

**ABSTRACT**

***Objective(s)***: Enterotoxigenic *Escherichia coli* (ETEC) is known as the most common bacterial causes of diarrheal diseases related to morbidity and mortality. Heat-labile enterotoxin (LT) is a part of major virulence factors in ETEC pathogenesis. Antigen entrapment into nanoparticles (NPs) can protect them and enhance their immunogenicity.

***Materials and Methods:*** In the present study, recombinant LTB protein was expressed in *E. coli* BL21 (DE3) and purified by an Ni-NTA agarose column. The protein was entrapped in PLGA polymer by the double emulsion method. NPs were characterized physicochemically and the protein release from the NPs was evaluated. ELISA assay was performed for investigation of raised antibody against the recombinant protein in mice. The anti-toxicity and anti-adherence attributes of the immune sera against ETEC were also evaluated.

***Results:*** It showed the successful cloning of a 313 bp DNA fragment encoding LTB protein in the pET28a vector. Over-expression in BL21 (DE3) led to the formation of corresponding 15.5 kDa protein bands in the SDS-PAGE gel. Western blotting by using anti-CTX confirmed the purified LTB. Protein-entrapped NPs had a spherical shape with the size of 238 nm mean diameter and 85% entrapment efficiency. Immunological analyses showed the production of a high titer of specific IgG antibody in immunized animals. The neutralizing antibody in the sera of immunized animals was approved by GM1 binding and Ileal loop assays.

***Conclusion:*** The results indicate the efficacy of the entrapped LTB protein as an effective immunogen which induces the humoral responses.

► *Please cite this article as:*
Kordbacheh E, Nazarian Sh, Sadeghi D, Hajizadeh A. An LTB-entrapped protein in PLGA nanoparticles preserves against enterotoxin of enterotoxigenic *Escherichia coli*. Iran J Basic Med Sci 2018; 21:517-524. doi: 10.22038/IJBMS.2018.27017.6609

## Introduction

Gastroenteritis diseases cause about 3 million deaths worldwide, annually. While Viruses are the most common agents of gastroenteritis in developed countries, bacterial agents are prevalent in developing countries (1, 2). Enterobacteriaceae, are large groups of enteric, non-spore forming bacteria that are considered regular inhabitants of the intestine in both animals (e.g. piglets and calves) and humans (3). Several strains of enterotoxigenic *Escherichia coli* (ETEC) are currently recognized as some of the main bacterial causes of neonatal diarrhea, which is the second leading cause of infant mortality in developing countries (4, 5). ETEC can adhere to the small intestine by colonization factors and produce enterotoxins in a noninvasive manner. ETEC can secrete heat-stable enterotoxins (STa) and/or LTs. STa toxin can remain active at temperatures up to 100 ˚C, while LTs are inactivated at this temperature. Secretion of STa, LT, or the combination of both will cause diarrhea. LT enterotoxin is composed of two subunits: heavy chain (LTA) and light chain (LTB)(6, 7). LTA is the toxic segment that because of its ADP-ribosyl transferase activity, is the cause of diarrhea. This protein activates adenylate cyclase via increasing the concentration of cyclic AMP in the intestinal cells and induces dehydration. LTB is an 11.6 KD peptide that assembles as a homopentamer of 55 KD, which joins to A subunit by non-covalent bonds and then, binds to $GM_1$ ganglioside receptors on the surface of enterocytes (8). So far, some antigens are introduced as candidate immunogens. However, currently, there is no efficient and licensed vaccine against the bacteria that is able to present a comprehensive protection toward different ETEC strains. Poor efficiency of these vaccines is the short period of protection. According to the published studies, the immunization lasts for about 3–6 months (9, 10). Therefore, several studies have shown that nano-carriers can improve immune responses and also increase erosion release. Polylactic-co-glycolic acid (PLGA) nanoparticle is a desirable polymer that has FDA and European Medicines Agency (EMA) approvals. Its safety refers to the fact that the lactic acid and glycolic acid monomers are readily metabolized in the Krebs cycle (11). PLGA monomers ratio usually defines the structures and specifications of the nanoparticles, and the monomers ratio determines the release of the entrapped protein. A 75:25 ratio defines a polymer that includes 75% lactic acid and 25% glycolic acid. Knowing that PLA has a lower degradation rate, it can be inferred that the mentioned ratio of 75:25 can release antigens more slowly (12). In this study, we entrapped one of the most important virulence factors of ETEC (LTB) in

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PLGA, to investigate the efficiency of this formulation in the protection of immunized animals against ETEC.

## Materials and Methods

### *Bacterial strains, plasmids, and media*

The bacterial strains used in this study were enterotoxigenic *E. coli* and *E. coli* strain BL21 (DE3). The pET28 plasmid was taken from Novagen (USA). Luria-Bertani (LB) broth and LB agar, which were used to cultivate the bacterial strains were from Merck (Germany).

### *Cloning of eltb gene*

The sequence of the *ltb* gene was adopted from GenBank with the accession number of M17874. The primer pair was designed by using the Oligo software. The sequences of forward and reverse primers were TGCAGAATTCGCTCCTCAGTC & TTACAAGCTTCTAGTTT-CCATACTGATTG, respectively. The ETEC strain was cultured in LB broth, and bacterial genome was extracted via the CTAB-NaCl method. The PCR was carried out in a reaction composed of 50 ng/ml of DNA, 4 pM of forward and reverse primers, 0.4 mM of dNTP mix, 3 mM of $MgCl_2$, 1X PCR buffer and 4U Taq DNA polymerase (Cinnagen Tehran, Iran) in a volume of 25 µl. Thermocycler stages set for 94 ˚C for 5 min as initial denaturing and 30 cycles of 94 ˚C (30 sec), 58 ˚C (30 sec) and 72 ˚C (60 sec) respectively for denaturing, annealing and a final 5 min at 72 ˚C. PCR purification kit (Bioneer Daejeon, Korea) was applied to purify PCR products. pET28a vector and PCR products were digested with *Eco*RI and *Hind*III restriction enzymes and purified before proceeding with the ligation reaction. Ligation was performed with T4 DNA ligase overnight at 14 ˚C, and the mixture was transferred into fresh prepared competent *E. coli* BL21 (DE3) cells. Transformed clones were confirmed by colony PCR and restriction digestion analysis.

### *Expression and purification of recombinant protein*

For expression, the recombinant clones were grown in LB broth with 70 µg/ml of kanamycin with agitation overnight at 37 ˚C. The culture was used to inoculate 100 ml of LB medium containing 70 µg/ml kanamycin and the culture was grown at 70 ˚C to an optical density of 0.5 at 600 nm. Expression was induced by the addition of IPTG (Isopropyl-β-D-1-Thiogalactopyranoside) to a final concentration of 1.0 mM at 37 ˚C for 4 hr. The cells were centrifuged at 1500×g for 5 min at room temperature. The pellets were resuspended in lysis buffer ($NaH_2PO_4$ 100 mM, Tris-HCl 10 mM, urea 8 M), and sonicated 5 times for 1 min at 30 sec intervals. After centrifugation at 12000 rpm, 4 ˚C for 20 min, the supernatant was collected for further analysis on 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

The protein was purified by batch purification of His-tagged proteins from *E. coli* under denaturing conditions (Qiagen). The supernatant was poured onto the affinity column. The column was washed with a washing buffer (100 mM $NaH_2PO_4$, 8 M Urea, 10 mM Tris-HCl. pH=6.3) and bound protein was eluted using 2 ml of elution buffer (100 mM $NaH_2PO_4$, 8 M Urea,10 mM Tris-Cl, pH=4.5) followed by 1 ml of 20 mM MES (2-(N-morpholino) ethane sulfonic acid) buffer. SDS-PAGE electrophoresis monitored the purified protein, and the denaturant agent (8 M urea) was removed by stepwise dialysis.

### *Western blot analysis*

For immunoblot analysis, proteins were electrophoretically transferred onto a nitrocellulose membrane (Roche, Germany) using transfer buffer containing 39 mM glycine, 48 mM Tris-base, 0.037% SDS, and 20% methanol. The membrane was blocked with 5% skimmed milk in PBST (13 mM NaCl, 2.7 mM KCl, 4.3 mM $Na_2HPO_4$ and 0.05% (v/v) Tween-20) overnight at 4 ˚C. The nitrocellulose membrane was washed three times with PBST and incubated for 1 hr with anti-CTX (Sigma, Germany) at 1:5000 dilution. Following washing the membrane with PBST, anti-rabbit IgG conjugate (Dako, Denmark) was added and incubated for 1 hr at 37 ˚C. Finally, detection was carried out using an HRP staining solution containing DAB (Sigma, Germany). Chromogenic reaction was halted by rinsing the membrane twice in distilled water.

### *Double emulsion method for preparing nanoparticles*

Since proteins are hydrophilic macromolecules, w/o/w (water/oil/water) method as a customary solvent evaporation procedure is preferred (13). According to this approach, a (polylactide-co-glycolide) polymer with the ratio of 75PLA:25PLGA (66000-107000 Mw) was used as the entrapment agent. Temporarily 30 mg of PLGA powder was dissolved in 4 ml of dichloromethane dissolvent on a stirrer. One ml of the protein solution with concentration of 1 mg/ml was added to the mixture drop-wisely. The optimized amount of protein was obtained through loading of the BSA protein onto the mixture. Emulsification was accomplished via ultrasonication (0.5 cycles pulse and amplitude of 50 for 20 sec, three times). Then, the new emulsion was embedded into 8 ml poly-vinyl alcohol (PVA 2%w/v) as the cross-linker agent and homogenized in an ultrasonicator again. The final w/o/w double emulsion was appended to 20 ml of double-distilled water (DDW) and stirred for at least 1 hr. Hence, the first solvent evaporated, resulting in formation of NPs, which were collected via centrifugation.

### *Physico-chemical properties*

Before the final centrifugation in the previous step, the suspension was diluted ten times and then one drop was deposited on an aluminum film and dried at room temperature, so that a uniform layer of NPs was formed. Particle size was analyzed by scanning electron microscopy Vega3 (TESCAN Vega LMU, USA). Besides SEM analysis, particle diameter and its distribution in solution (PDI) was measured by the dynamic light scattering (DLS) method, also the zeta potential (ζ) of the NPs was investigated. For this purpose, 1 ml of final w/o/w solution was prepared as mentioned above, then, sonicated at the 50% amplitude and 0.5 sec pulse cycle for 1 min. Finally, DLS system optical characteristics such as λ-max and the reflective index were adjusted according to PLGA polymer and DDW dissolvent (DLS, Malvern, MAL1001767).

### *In vitro release of protein from the NPs*

Two phenomena are proposed for protein release

**Table 1.** Summary of immunization protocols. The groups of subcutaneously and intraperitoneally immunized mice with free LTB and entrapped in PLGA

| Groups | Administration | Antigen (dosage) | Number of administrations | Adjuvant | Route of immunization |
|---|---|---|---|---|---|
| 1 | Entrapped in PLGA | LTB (20µg) | 4 | Freund's | Subcutaneous |
| | | | | Freund's | Intraperitoneal |
| | | | | - | Subcutaneous |
| | | | | - | Intraperitoneal |
| 2 | Free protein | LTB (20µg) | 4 | Freund's | Subcutaneous |
| | | | | Freund's | Intraperitoneal |
| | | | | - | Subcutaneous |
| | | | | - | Intraperitoneal |
| 3 | Control | PBS | 4 | - | Intraperitoneal |

from the NPs. The first, simulation for *in vivo* release, examined by dissolving the PLGA pellet containing 3 mg protein in 0.5 ml simulated body fluid (SBF) and incubated at 37 ˚C in a thermostatic shaker (200 rpm). Afterward, for an approximately forty-day period, every three days it was centrifuged (10000 rpm, 5 min), protein concentration in the supernatant was measured, and the fluid was exchanged with a fresh one. The second, high burst release, which was estimated by resolving entrapped protein in SDS 0.1 M and NaOH 5% solution and shaking at 37 ˚C with 200 rpm, was obtained. In addition, the supernatant proteins were loaded on SDS-PAGE for comparison.

### *Entrapment and release parameter*

The protein NPs content was analyzed in the last stage of nanoparticle preparation as previously reported. After final centrifugation, the entrapped protein was precipitated in the bottom of a falcon, the supernatant was separated and its protein amount measured via the Bradford method. The Bradford blank solution was chosen from a similar PLGA NPs preparation without any entrapped protein. The final solutions were lyophilized in a freeze-drier system to get the weight of the entire contents (CHRIST Alpha 2-4, Germany). The following formula was used to calculate the entrapment efficiency (EE), Particle Yield (PY), and Loading Capacity (LC):

Also, various kinetic models, such as zero order, first order, Hixson-Crowell, Korsmeyer-Peppas, Higuchi (square root), and Weibull model square root (Higuchi) were fitted with protein release curves by the KinetDS software. Mean dissolution time (MDT) was another benefit and a model-independent parameter was obtained via this software; the closest model to best interpret the highest determination coefficient ($R^2$) with the lowest RMSE (root mean square error) and AIC (Akaike's Information Criterion) (14, 15) the in vitro dissolution test becomes a standard tool for characterization of manufactured products. However, results of the dissolution test must be expressed in mathematical terms; this is realized by fitting various models to the cumulative dissolution curves. The models might be either mechanistic or empirical. The fitting process requires software (e.g., KinetDS.

### *Stability and antigenicity of the entrapped protein*

Antigenicity and structure conservation of the released protein from NPs was confirmed by Western blotting as described earlier. In this technique mouse anti-LTB antibody (developed in-house at our department) was applied to react with nitrocellulose-bound protein.

### *Immunization procedure*

Overall three mouse groups (20 g, 6–8 weeks old) were chosen for immunization experiments. Table 1 lists nine groups of animals inclusive of encapsulated LTB protein, free LTB with or without Freund's adjuvant. Every mouse received 20 ug protein every 14 days. Blood samples were taken for immunoglobulins and toxin examination ten days after each administration.

### *Determination of immunoglobulins responses to recombinant protein*

The enzyme-Linked immunosorbent assay (ELISA) method was performed for measuring IgG titers after each administration. Briefly, each well of the polystyrene microplates (Nunc) was coated with 5 µg LTB with carbonate/bicarbonate buffer and incubated at 4 ˚C overnight. After washing five times with PBST, wells were blocked with a 5% skimmed milk solution as the blocking buffer; after 2 hr incubation at 37 ˚C, wells were washed again and incubated with serially diluted serum and fecal samples. The wells were washed with PBS-T and anti-mouse IgG and IgA antibody were added as the secondary antibody and incubated for 1 hr at 37 ˚C. After washing, 100 µl of a citrate buffer containing 0.06% (W/V) of O-phenylene diamine dihydrochloride (OPD) (Sigma) and 0.06% (V/V) hydrogen peroxide were added to each well and incubated at room temperature for 15 min. The reaction was stopped by addition of 100 µl of 2 M $H_2SO_4$ and the results were read on a microplate reader (Bio-Rad) at the wavelength of 492 nm.

### *Toxin neutralization assay*

To assess the ability of serum samples of immunized mice to neutralize the LT toxin, ileal loop assay and $GM_1$ inhibition assay were conducted. For $GM_1$ inhibition assay, serially diluted serum collected from immunized mice was mixed with LT and incubated at 37 ˚C for 1 hr. The mixture was then added to 5 µg/ml of $GM_1$ (Sigma) coated well plates and incubated at 37 ˚C overnight. To remove nonbinding toxins, plates were washed three times with PBST. Uncovered nonspecific binding sites were blocked by the addition of a blocking buffer containing 3% BSA at 37 ˚C for 1 hr. Plates were emptied and washed again as described above.

The toxin bound to $GM_1$ was detected with 1:2000 dilution of rabbit anti-cholera toxin antiserum (Sigma Aldrich) and 1:2500 dilution of goat anti-rabbit immunoglobulin conjugated to horseradish peroxidase (Dako, Roskilde, Denmark) using $H_2O_2$ and OPD (O-phenylenediamine dihydrochloride) as substrates. The reaction was stopped by addition of 2 M $H_2SO_4$. The absorbance of the solutions was measured at a wavelength of 492 nm. Ileal loop assay was performed according to the De and Chatterjee method (1953) (16). ETEC strain cultivated from single colonies in 30 ml of CAYE broth [2% Casamino acids, 0.6% yeast extract, 43 mM NaCl, 38 mM $K_2HPO_4$, 0.1% trace salt solution consisting of 203 mM $MgSO_4$, 25 mM $MnCl_2$, and 18 mM $FeCl_3$] was incubated overnight at 37 ˚C. Mice were starved for 24 hr before surgery. The animals were anesthetized with intraperitoneal ketamine (40 mg/kg) and xylazine (8 mg/kg), and 3 cm ligated ileal loops were constructed. ETEC bacteria at $1\times10^8$ CFU/ml

**Table 2.** Kinetic parameters of release profile Kinetic parameters of release profile of recombinant LTB entrapped in PLGA nanoparticles

| Statistical parameter | MDT [day] | Zero-order | First-order | Higuchi model | Korsmeyer-Peppas | Hixson–Crowell | Weibull model |
|---|---|---|---|---|---|---|---|
| Value | 2.076 | $R^2$: .9926 | $R^2$: .2432 | $R^2$: .2935 | $R^2$: .988 | $R^2$: .8332 | $R^2$: .9981 |
| | | AIC: 1.36 | AIC: 1.62 | AIC: 9.63 | AIC: 2.31 | AIC: 6.03 | AIC: 2.37 |
| | | RMSE: 3.38 | RMSE: 3.48 | RMSE: 4.48 | RMSE: 4.55 | RMSE: 1.36 | RMSE: 4.63 |

concentration were incubated with mice serum for 30 min. The mixture was added to each loop created in the intestine. The mice were sacrificed 18 hr later and then, the ratio of fluid accumulation against loop length (g/cm) was calculated as an index of enterotoxigenicity.

## Results

### *Preparation and characterization of protein*

The *eltb* gene was cloned into a pET28a expression vector and was confirmed by PCR and restriction digestion analysis. Recombinant vector was transformed into the *E. coli* BL21 cells and protein expression was induced by IPTG. A 15.5 kDa protein band on SDS-PAGE showed a high level of recombinant protein expression (Figure 1A). The expression of recombinant protein was confirmed by reaction with the anti-CTX antibody with Western blotting (Figure 1B). Purification of the protein was carried out under denaturation conditions, and SDS-PAGE analysis revealed the presence of the recombinant protein in the eluted fraction (Figure 1C).

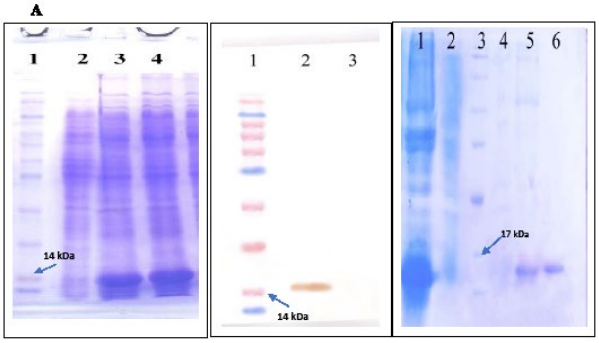


**Figure 1.** Analysis of expression, purification, and Western blot of recombinant protein. (A) SDS PAGE analysis of recombinant protein expression. Lane 1: protein weight marker (Vivantis). Lane 2: total protein of non-induced cells. Lanes 3, 4: total protein of induced bacteria. (B) Western blot analysis of recombinant LTB using anti-CTX antibodies. Lane 1: protein weight marker (Vivantis). Lane 2: recombinant protein. Lane 3: BSA (C) Purification of 6x His-tagged LTB under denaturing conditions and stepwise pH. Lane 1, Cell lysate; lane 2, flow-through; lane 3, protein weight marker (Cinnagen); lane 4, column washed with buffer C; lane 5, column washed with buffer D; lane 6, purified protein after elution with buffer E

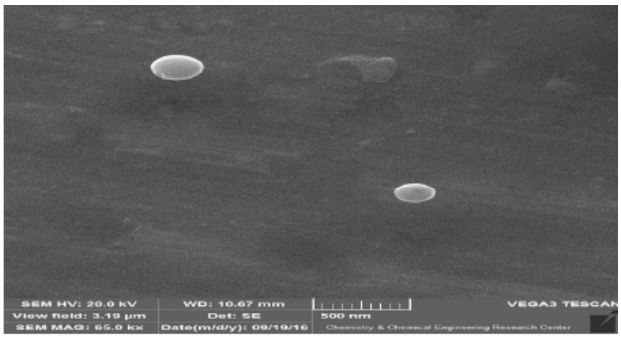


**Figure 2.** Scanning electron micrograph of PLGA nanoparticles entrapped with LTB. The NPs were prepared by the double emulsion method as described in Methods

### *Entrapment and dissolution characterization*

After preparation of NPs by an evaporation method (W1/O/W2-solvent), estimated LTB loading efficiency was 84.5± 5%. Also, it was approximately higher than 70%, during nanoparticle absorption set up with BSA protein. Furthermore, the particle yield and loading capacity percentage were measured as 88% and 3%, respectively. Afterward, the morphology of entrapping nanoparticle inspected approximately uniform and spherical by scanning electron microscopy (Figure 2). Moreover, the average size of NPs after coating with PVA was measured around 238 nm in reporting by "intensity". The mean zeta potential (ζ) was determined as -13.2 mV and -4.68 mV subsequently prior to and following protein loading, besides polydispersity index (PDI) for the solution of 0.362 (Figures 3A and B). *In vitro* release of LTB protein from the PLGA NPs in SBF exhibited a sustained release of protein, so that about 12% of total loaded antigen was released after a forty-day period (Figure 4). The zero-order kinetic model had the most similarity with the release profile. In this model, kinetic parameters consist of coefficients of determination measured as 0.9926 ($R^2$) with small error value for RMSE (3.38) and AIC (1.36). Also, MDT, as a model-independent parameter, was measured as 2.07 days (Table 2). Antigenicity of PLGA-entrapped antigen determined by SDS PAGE and Western blot analysis showed that the specific anti-CTX antibody recognized the released recombinant protein (Figure 5).

### *Antibody responses*

All animals were immunized with the entrapped and free protein except the control group, so animals were seen healthy and showing no signs of abnormal behavior excluding a brief inflammation after subcutaneous injection with the Freund's adjuvant. An individual antibody against LTB protein was assessed by using the ELISA technique as stated earlier. After vaccination, the mouse immune system produces significant IgG, especially in intraperitoneal administration, though the groups were not significantly different; also, inconsiderable IgA antibodies were measured in serum and fecal samples compared to the control animals. Approximately all entrapped experimental groups exhibited efficient IgG titer from a high concentration of serum unto 1/16000 rarefaction; however, microplate readers indicated entity of antibody even at 1/64000 dilution. IgG titer of entrapped LTB was examined after a forty-day period (Figure 6). This result measured for entrapped LTB, while immunization with free protein additionally indicated robust immune responses, of which some were better than those of the entrapped ones.

### *Toxin neutralization assay*

*In vitro* binding of LT toxin to the $GM_1$ ganglioside

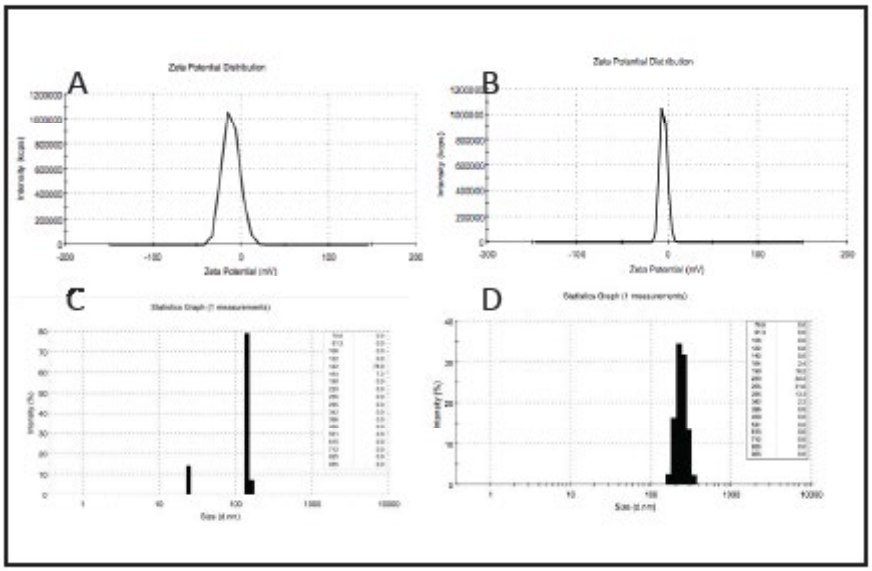


**Figure 3.** From the graphs, the zeta potential of control solution (PLGA coated with PLA) was measured -13.2 mV(A), also the zeta potential of NPs-LTB was estimated -4.68 mV (B). The charts show size statistic and its distribution in which size was 144 nm and 238 nm before (C) and after (D) protein entrapment, respectively (PDI was measured as 0.362 subsequent to protein loading)

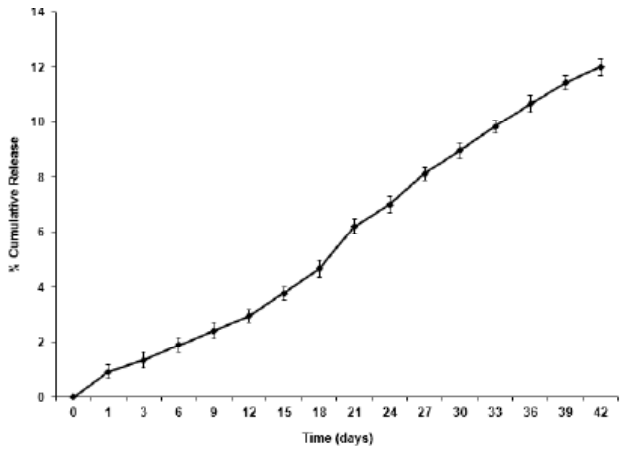


**Figure 4.** *In vitro* erosion release of entrapped LTB following a forty day period from the last antigen administration. The graph presented zero order kinetic model for release profile (approximately 12% of protein released during this time)

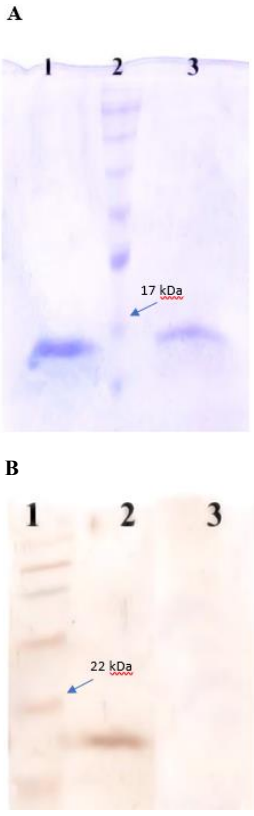


**Figure 5.** SDS PAGE and Western blot analysis of the released LTB antigen from PLGA nanoparticles. (A) Lane 1, protein LTB; Lane 2, protein weight marker (Cinnagen). Lane 3, the protein released from nanoparticles. (B) Lane 1, protein weight marker (Vivantis). Lane 2, LTB released from PLGA nanoparticles reacted with Anti-LTB; Lane 3, BSA (it is worth mentioning that LTB bond changed a little after treatment with SDS-NaOH solution)

was blocked as a result of subjecting antibodies from immunized mice to $GM_1$-ELISA (Figure 7). The inhibitory effect of the anti-LTB antibody on fluid accumulation induced by toxin was confirmed via the mice ileal loop assay. The fluid accumulation was observed in ileal loops 18 hr post infection with ETEC treated with non-immunized mice antibody. In contrast, infection with ETEC pretreated with immunized mice antibody was accompanied by the reduction of fluid secretion. A significant difference in reduction of fluid accumulation in ileal loops infected with ETEC pretreated with immunized mice antibody was observed in comparison with the control loop ($P$>0.05).

## Discussion

On average, bacteria cause 63% of gastroenteritis infections and studies have indicated ETEC is the most common agent in this regard. It is known that LT protein is a critical apparatus in terms of ETEC invasion besides its broad usage as an adjuvant (dmLT). Today, the challenges of vaccine development are not restricted to the selection of efficient and safe antigens; but the delivery system is crucial in this context. Recently, new vaccine delivery methods have been developed using

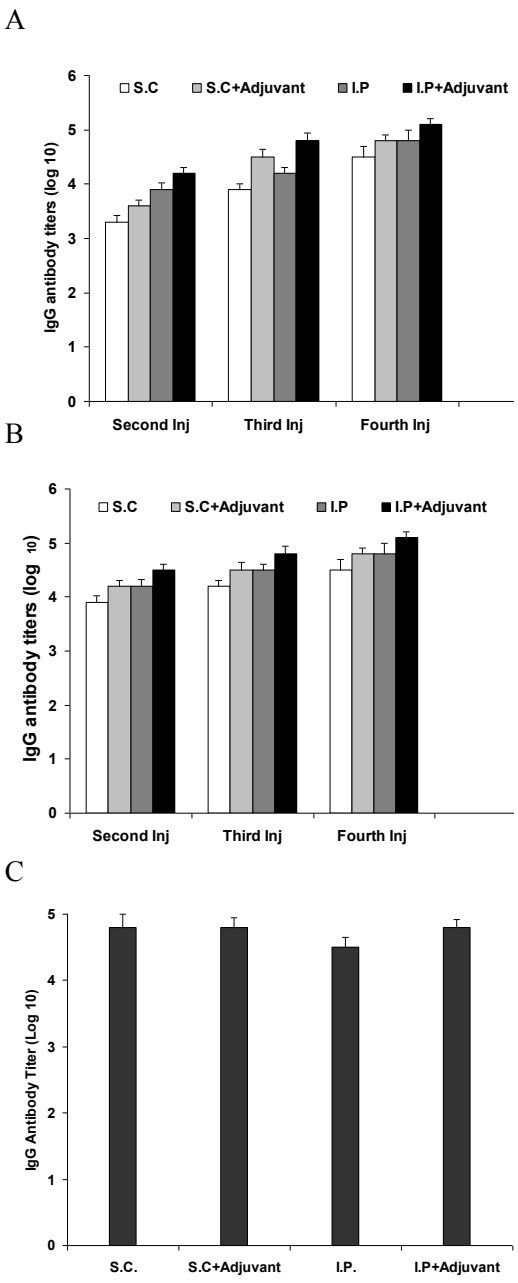


**Figure 6.** A: IgG titer of entrapped LTB diagrams demonstrated, each blood sample was collected a week after any administration. B: IgG diagram for immunization with free LTB. C: This diagram indicates IgG titer of the entrapped group, six weeks after the last injection, as an index of erosion release

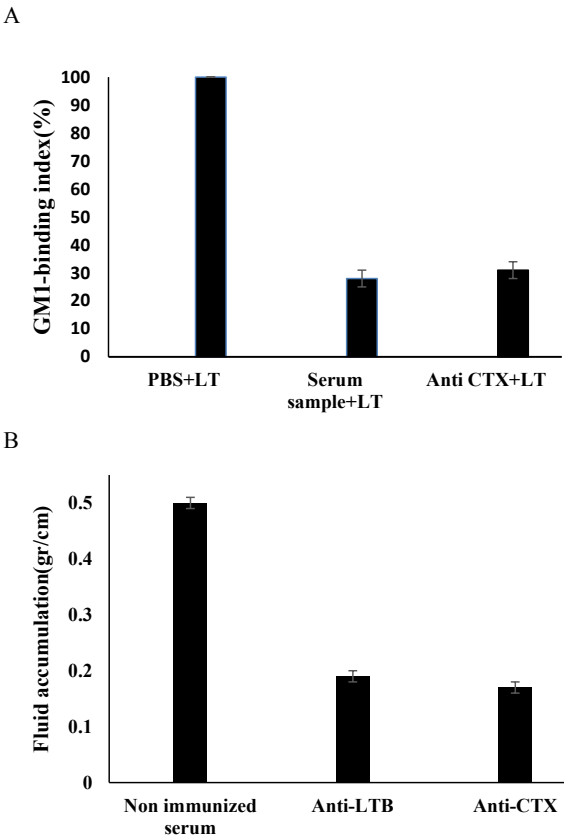


**Figure 7.** Toxin neutralization assay. (A) Effect of anti-LTB on the binding of LT to the GM1 receptor. The inhibition was determined using GM1 ELISA assay. The neutralizing index determined with OD492 obtained by GM1-ELISA. Values are expressed as mean ±SD (n=3). (B) Effect of anti-LTB on the reduction of fluid accumulation in ileal loops. The test was carried out as described in Materials and Methods, and the fluid accumulation ratio (g/cm) was calculated

NPs and biodegradable polymers like PLGA polymer (10, 17). So PLGA NPs with their biocompatible and biodegradable features were selected for overcoming accelerated exposure of the protein to immune system besides the adjuvant role of these NPs. It is worth mentioning that LTB has adjuvant property itself, but in recent years it was shown a combination of more than one adjuvant can motivate antigen presenting cells (APC) more than one level to boost immune responses. Among some volatile solution for the first aqueous phase in w1/o/w2 method, DCM was preferred to w1 for its better efficiency and broad usage. In the end, PVA as a nonionic surfactant was applied as the second emulsifier (covalent linker), attachments of which to hydrophobic PLGA can restrict poly formation (18, 19). -13.2 mV zeta potential confirmed polarization role of PVA as a covalent linker, especially since the immune system recognizes hydrophobic particles as foreign and eliminates them by the reticuloendothelial system (RES) (20). Moreover, its less negative charge is due to PVA presence, a 2% w/v concentration of which is enough for overcoming poor encapsulation efficiencies by itself since small proteins have to escape tendency into the w2 phase during the entrapment (21). LTB is expressed as inclusion bodies so it was necessary to dissolve this protein by a urea-containing buffer. For removal of urea, in-situ refolding was applied. To do this, 1 ml of the protein solution was applied to the NI-NTA column. Then the column was washed by using buffers with low concentration of urea. At the final step, the washing buffer had no urea, instead, it had imidazole for separating the protein from the column. Entrapment efficiency of 85% is desirable and gives us a concept about the cargo percentage which is entrapped or adsorbed into NPs while a range of 6–90% (EE) is observed in the literature by PLGA entrapment. Moreover 3% loading capacity helps us to deal with NPs following their separation from the medium and to comprehend their cargo content (22–24). The second pitfall of NPs entrapment after loading efficiency refers to high burst release. This

phenomenon occurs due to the presence of antigens located near the surface of the NPs. Therefore, exterior antibody release measured about 1% subsequent to first overnight incubation in SBF, and it corresponds to a priming immunization dose logically (25, 26). In terms of release profile, a favorable kinetic model has the maximum value for determination coefficient ($R^2$) with the minimum amount of RMSE (root mean square error) and AIC (Akaike's Information Criterion) parameters (27). Thus the zero-order model had the most similarity with the observed diagram because of its high R square and lowest AIC and RMSE error. It seems this linear release profile is due to copolymer ratio (75% PLA, high-density) which causes slower release. So, it generally provides maximum therapeutic value with minimum side effects because of regular and linear dissolution profile (28, 29). Anti-CTXB and LTB antibodies confirmed the nature and structure of the protein at different levels, including LTB expression, NPs release profile, and neutralization assay. The purpose of both intraperitoneal and subcutaneous administration was to investigate the route of immunization in immune response, with the view that the antigen release is slower through subcutaneous route, though there were no significant differences. Also, other time points of ELISA results exhibit no significant differences between positive Freund's adjuvant and nanoparticulated LTB administration (without Freund's adjuvant), which indicates enough costimulator motivation and erosion release of NPs implicitly. Thus, the ELISA results show noteworthy titer of antibody despite erosion release of nanoparticulated protein, and secondary adjuvant is not required. As mentioned above the high-density PLGA leads to a very gradual protein release, 12% after one month. The similarity between free and encapsulated antibody titer may arise from this point.

In terms of these same responses in test groups, neutralizing and ileal loop assays applied were based on one sample (30, 31). The high affinity of $GM_1$ for the heat-labile toxin of ETEC and cholera toxin formed the basis of an ELISA assay of this interaction. LT neutralizing activities indicate anti-LTB antibodies can recognize native forms of LTB in holotoxin and prevent LT binding to $GM_1$-ganglioside. Immunological protection against the LT toxin from ETEC may be due to the effect of an antibody against LTB on prevention of LT toxin binding to cellular $GM_1$. Ileal loop assay was performed to confirm production of anti-LTB antibodies in mice. So, statistically significant reduction in intestinal fluid accumulation ($P>0.05$) was detected upon pretreatment of ETEC cells with antibodies. This data demonstrates that antibody against recombinant LTB can recognize the native form of LTB in holotoxin and possess the ability to block the LT binding to its receptors and cause a reduction in intestinal fluid accumulation. The results demonstrate that antibodies prepared against the LT toxin blocked the toxin activity in ileal loops and these assays have no significant differences with some immunogen candidates, including the LTB protein (32–34).

## Conclusion

These results further support the application of 75:25 PLGA nanoparticles with favorable features such as low-burst release (zero order kinetic) and adjuvanticity role as a safe carrier for LTB delivery, thereby protection against ETEC.

## Acknowledgment

The authors wish to thank Deputy of Research, Imam Hossein University, Tehran, Iran for financial support.

## Conflicts of Interest

Authors declare no conflicts of interests.

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