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RESEARCH PAPER

Evaluation of mPEG-PLA nanoparticles as vaccine delivery system for modified protective antigen of *Bacillus anthracis*

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ABSTRACT

Objective(s): *Bacillus anthracis* is the cause of the fatal anthrax. Available anthrax vaccines have low stability and require multiple injections in order to be effective. Poly lactic acid (PLA) has been approved as a biodegradable and biocompatible polymer for drug and vaccine delivery applications. The purpose of this study is to evaluate the antibody titer against the protective antigen recombinant protein (PA63) encapsulated by the mPEG-PLA double-block copolymers and to compare with the non-encapsulated PA63.

Materials and Methods: To attain this purpose, to start, the desired protein was purified and confirmed and then PA63 was encapsulated with mPEG-PLA double-block copolymers using a water- oil- water solvent evaporation method. Produced nanoparticles was characterized in terms of morphological specifications using scanning electron microscopy, size and polydispersity index using dynamic light scattering and zeta potential using a zeta seizer. The synthesized nanoparticle antigenic content and also its antigen release profile was measured. In the following, the nanoparticles containing antigens (PA63-NPs), blank nanoparticles (mPEG-PLA- NPs), PA63 and adjuvant control were injected subcutaneously to mice and the IgG polyclonal antibody titr was measured by indirect ELISA. Finally to evaluate biocompatibility and toxicity, synthesized nanoparticles were investigated by cell culture testing.

Results: The results of this study showed that the synthesized nanoparticles are of good quality. ELISA results showed that antibody production titr in mice receiving PA63-NPs was higher than those receiving the PA63 ($P<0.05$). Cell culture results revealed that the synthesized nanoparticles have no toxicity.

Conclusion: The findings of the study indicated that the obtained nano vaccine formulations had a higher ability than non-encapsulated recombinant proteins to stimulate the immune system of animal, and that PLA could be used as an appropriate carrier for an effective, stable, safe and biodegradable engineered recombinant vaccine against anthrax.

Keywords: *Anthrax, Protective antigen, Vaccine, mPEG-PLA*

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INTRODUCTION

Anthrax is a common disease between humans and livestock (zoonosis) that is caused by *Bacillus anthracis* in the *Bacillaceae* family [1, 2]. Extremely easy production and dispersion are the features of this highly pathogenic bacteria. The fatality rate in inhalational anthrax is 45–90% even when the anthrax gets diagnosed early and followed by an aggressive antimicrobial schedule [3]. Vaccines against *Bacillus anthracis* are one way to protect humans and animals against this bacterial disease [4, 5]. The first anthrax vaccine was developed by Pasteur in 1881. The immunity created against

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anthrax occurs as a result of neutralizing the activity of anthrax toxin [6]. PA is a very important component of anthrax toxin, with its gene located on the plasmid POX1. Regarding the role of PA, it has been widely studied as a candidate for vaccine [6-8]. The current anthrax vaccines are anthrax vaccine adsorbed (AVA) and anthrax vaccine precipitated (AVP) [9]. But the long-term vaccination program for vaccines (6 vaccinations in 18 months) limits its use, so a new generation of anthrax vaccines is needed. In recent years, the interest in using biodegradable polymer nanoparticle systems for drug delivery applications has been rising [10-12] and could act as a remedy for this problem.The encapsulation in such polymer nanoparticles have been shown to provide stability, sustained release controlled and decreasing the need of boosters [13].

Poly-lactic acid (PLA) is a FDA approved, biodegradable and biocompatible polymer [14-16]. The addition of polyethyleneglycol (PEG) to PLA results in the lowest zeta potential, higher drug entrapment, lack of absorption of plasma proteins and thus a higher durability in the bloodstream [17]. Due to PA's inability to tolerate conditions and low immunogenicity as a candidate for vaccine, PA was encapsulated in PLA microspheres and its results were compared with non-encapsulated PA [18]. Considering the research done and the available data of these facts, we assume that the PA63 nanoformulation can act as a more effective vaccine alternative without the need for adjuvant and multiple booster doses. In this study, we first investigated the potential of PA63-NPs as a carriers of an anthrax vaccine in increasing the immune response and in addition we examined the toxicity and biocompatibility of the prepared nanovaccine. The results of this study showed that the formulation of the resulting nanovaccine has a high ability to stimulate the immune system of the experimental animal and the nano-formulation can be obtained as an effective, stable and biodegradable recombinant vaccine suggested for further investigation.

MATERIALS AND METHODS

Chemicals and animals

The chemicals used in this study were from Merck, Kiagen and Fermentas [19]. Sterile deionized water was used to make all buffers. The secondary antibody conjugate to HRP against mouse immunoglobulin and Anti-HisTag antibody from Razi Biotechnology Company were purchased. The mice (Mus musculus) from 8 to 10 weeks of age were also provided by the animal maintenance and reproduction center of Baqiyatallah university of medical sciences.

Mice were kept in 12 hr light-dark period at 20 °C ± 2 °C under conditions of free access to food and water.

Polymer synthesis

The diblock copolymer mPEG-PLA was synthesized by the ring-opening polymerization method, as described by Garc´ıa et al [20]. Briefly, to start the polymerization required amounts of D,L-lactide (Acros Organics), monomethoxy poly(ethylene glycol) (mPEG) (Mw 5000) (Sigma-Aldrich), an Sn(Oct)2 (Sigma-Aldrich) after degassing under vacuum for one hour, were heated to 140 °C. After 18 h, the polymer was cooled and dissolved in chloroform and precipitated. Synthesized double-block copolymers were dried under vacuum at 50 °C for 24 hr. The molecular weight of the mPEG-PLA copolymer used in this study determined by gel permeation chromatography (GPC) and the copolymer composition was determined by proton nuclear magnetic resonance spectroscopy (1H-NMR) (YOUNG, YL Instruments, Korea).

Purification of recombinant protective antigen (PA63)

After expressing PA63 in *E. coli*, the produced antigen purified using nickel column affinity chromatography (ShineGene) [19].

Preparation PA63-NPs

PA63-NP nanoparticles were prepared by PA63 encapsulation in double-block mPEG-PLA copolymers using w/o/w solvent evaporation (21). First added 100 mg of mPEG-PLA to 2 ml of THF. After complete dissolution, 1, 2 and 3 mg purified protein added an aqueous phase as a drop of dye into an organic phase. To prepare the blank nanoparticles were used only from phosphate buffered saline (PBS, pH= 7.4). W/O emulsion sonicated with a strength of 75% for 60 seconds (Dr.hielscher, germany).

In all the steps we used sonicate, the mixture

was kept inside the ice. The sample was then added to 1 ml of 1% Polyvinyl alcohol (PVA).Then the sonicate (75% strength for 60 seconds) was used to obtain the W/O/W emulsion. The emulsion was mixed with magnetic stirrer for two hours to evaporate the organic phase and increase the strength of the nanoparticles. Then centrifugation (Sigma, usa) 13000rpm for 10 min was used to remove PVA and the resulting nanoparticles were washed with sterile deionized water and for three hours at 0.25 milligrams pressure and 80 minus temperature Celsius degrees lyophilization (Christ, Germany).

Determination of PA63 loading capacity, entrapment efficiency and in vitro release profile

The protein content loaded in nanoparticles measured by dissolving 20 mg of prepared nanoparticles in 5 ml of tetrahydrofuran (THF) and then vortexed and two hours' incubation at 37 Celsius degrees and then centrifuged 15 min at 13000 rpm, and finally the amount of protein in the supernatant was measured by UV/VIS spectrophotometer using the following formula.

Encapsulation efficiency (%) = (Total amount of encapsulated PA63 in NPs (mg)) / (Total amount of PA63(mg)) * 100

Loading Capacity (%) = (Total amount of encapsulated PA63 in NPs (mg)) / (Total amount of NPs (mg)) * 100

Quality of PA63 after encapsulation

Same amounts of PA63 before encapsulation and released antigen from PA63-NPs and expressed PA63 used in order to evaluate the quality and stability of the antigen during the encapsulation steps using 12% SDS-PAGE with sample buffer.

Release kinetics of PA63

The release kinetics of synthesized nanoparticles using PA63-NPs nanoparticles were prepared in 10 ml of PBS at pH 7.3 and stored at 37 °C. The obtained solution centrifuged at intervals (4h, 7 d, 14 d, 21 d, 28 d) (13000 rpm for 10 min) and the amount of protein in the supernatant was measured by UV/VIS spectrophotometer. All the release tests were performed in triplicate and the results were presented as the average data with standard deviations.

Morphological characterization and surface structure of nanoparticles

The prepared nanoparticles were diluted in 0.1 mg/ml concentration and used. The morphological characteristics of nanoparticles investigated by the Scanning Electron Microscope KYKY SBC12 (China).

Determination of size and zeta potential of nanoparticles

Zeta potential of nanoparticles measured by electrophoretic mobility using zetasizer, polydispersity index (PdI) and nanoparticle size were measured using Dynamic Light Scattering (DLS) (Malvern Instrument, UK) (wavelength 633 nm) solvent (water).

Mice immunization and measuring of PA63-NP and PA63 IgG titers

For this purpose, we used 8 to 10 weeks male mice as test and control. each group contains 5 mice and the groups included the recombinant antigen (PA63) with adjuvant, blank NPs (mPEG-PLA-NPs), encapsulated PA63 (PA63-NPs), and control group PBS with adjuvant. Injections were performed subcutaneously four times, first injection 20, second 15, third 10 and fourth 10, micrograms with incomplete adjuvant, in respectively at 1, 14, 28 and 42 days. Blood sampling was done on days 21, 35 and 50. After separation of sera, was saved at -20 ° C temperature. To study IgG antibody titer was used by indirect enzyme linked immunosorbent assay (ELISA) [22]. For this purpose of 96-well plates (SPL, South Korea) was used and results were specified using Biotech Epoch microplate spectrophotometer (USA).

Cytotoxicity assay

In vitro cytotoxicity of PA63-NPs was evaluated on the epithelial cell line of african green monkey kidney (Vero) by standard MTT test with three replications [19]. Vero cell line was purchased from the center of genetic resources (Iran). Dimethyl sulfoxide (DMSO) and trypsin-EDTA, 3-(4, 5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) from Sigma-Aldrich (USA), cell culture media (DMEM) Gibco (USA). Other materials used Prepared and obtained from Merck (Germany). After preparing the cells and ensuring the absence of contamination and counting the cells, Vero cells

Table 1. Characterization of mPEG-PLA diblock copolymers

^{1}HNMR				
LA/EG in feed	LA/EG in Product [a]	M_n [a]	M_n [b]	M_w / M_n [c]
1.5	1.28	11400	17000	1.14

Mn: Number average molecular weight. Mw: Weight average molecular weight. PDI: Polydispersity index. a Determined from the integration of 1H NMR peaks. b Determined by GPC

were seeded in 96-well plates containing DMEM and FBS 10%. Test sample including PA63-NPs solution were sterilized with 0.2-micron filter and were used at concentrations of 10, 20, 40 µg/ml. It was then incubated for 24 hr at 37 ° C, 5% CO_2 and 95% humidity. Then 5 microliter of MTT solution at a concentration of 5 mg/ml was added to each well and the plates were incubated for 2 h at 37 °C. The media containing MTT was evacuated and then 100 µl of DMSO solution was added to each well and the plates were incubated for 2 hr in darkness at room temperature. Absorption values were specified using BioTek Epoch microplate spectrophotometer (USA) at 570 nm [23]. All experiments was performed with three replications. The viability of cells was calculated as follows:

Cell viability (%) = (OD absorbance of treated cells) / (OD absorbance of control cells) * 100

Finally using SPSS software (Ver. 16) and one-way ANOVA for statistical analyses, and the results were presented as mean ± SD at 5% significance level.

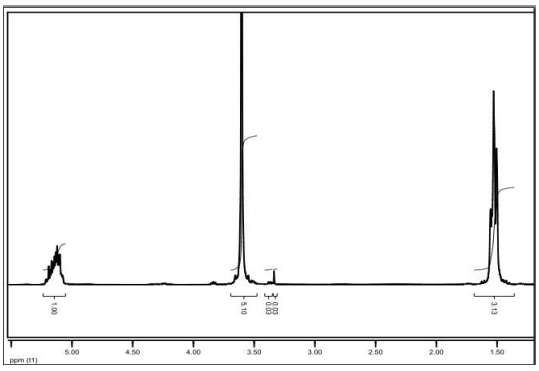

Fig 1. ^{1}H NMR spectrum of PLA-PEG diblock copolymers

Statistical analysis

Finally, SPSS software (Ver. 16) was utilized for calculating the differences between control and treated groups. The statistical analysis of the samples with three replications was undertaken using one-way ANOVA, and the results were presented as mean ± SD at 5% significance level.

RESULTS

Polymer synthesis and characterization

The molecular weight of the Synthesized mPEG-PLA copolymer was (Mn: 12 kDa). The structure and characterize of diblock copolymers confirmed by 1H NMR spectra (Fig 1) and GPC (Table 1).

Characteristics of PA63-loaded mPEG-PLA-NPs

Morphological study of NPs

Morphological study of NPs with and without PA63 was performed by SEM. The SEM images showed the spherical particle shape and surface structure of the nanoparticle (Fig 2). These images also confirm the results of the DLS for the average size distribution of nanoparticles.

Size and zeta potential of NPs

The particle size and zeta potential of NPs measured using DLS. The results indicated that the blank or protein-free nanoparticles showed 162 nm and polydispersity index indicated 0.034 and after loading PA63 nanoparticles containing protein showed a size of 233 nm

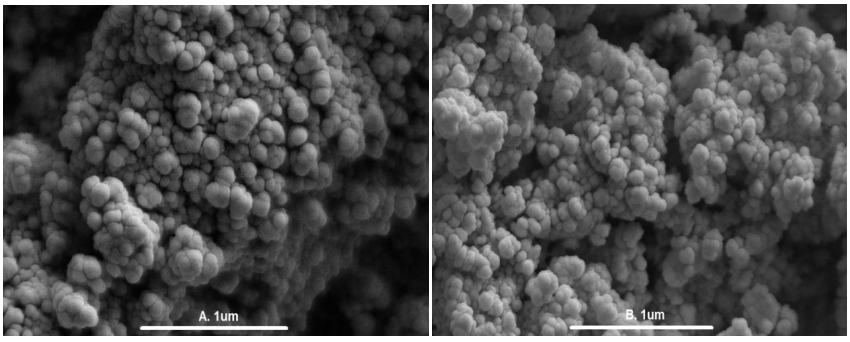

Fig 2. Scanning electron micrographs of NPs. A: PA63-NPs and B: mPEG-PLA-NPs. (scale bar is 1 um). magnification, 40.0 kx

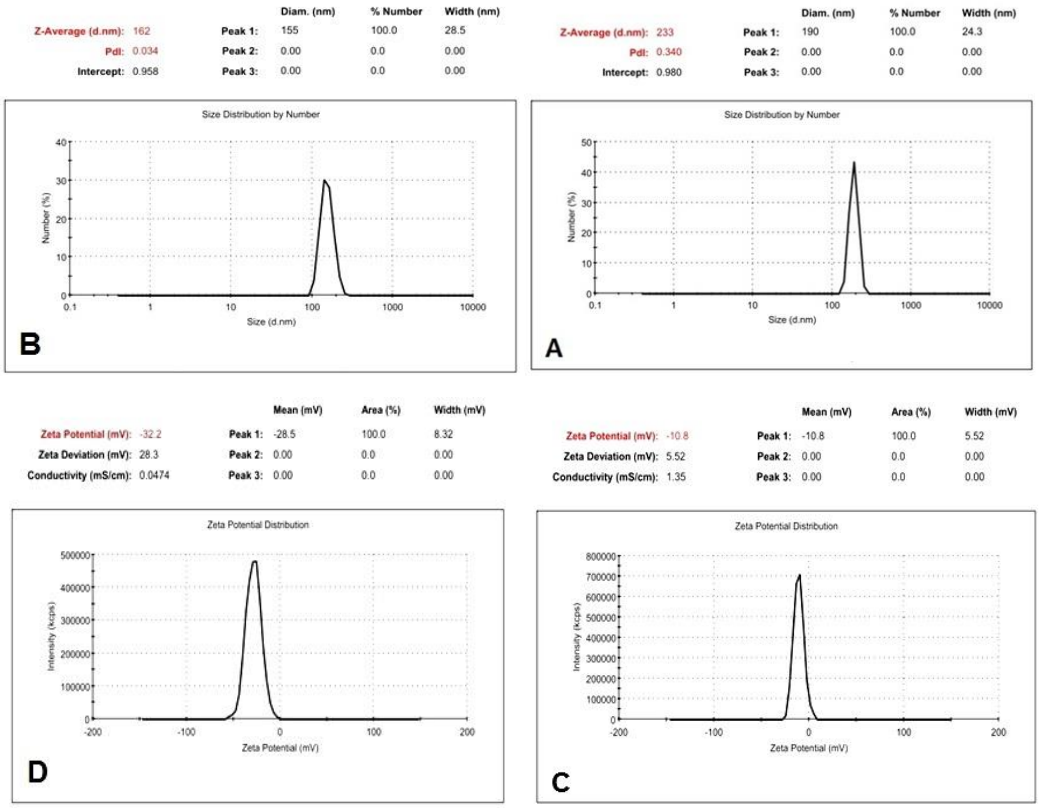

Fig 3. DLS and zetasizer data for prepared nanoparticle suspended in the dispersant (water). A: The z-average of PA63-NPs, 233 (d.nm) and polydispersity index, 0.340. B: mPEG-PLA-NPs, 162 (d.nm) and 0.034. C: The zeta potential of mPEG-PLA-NPs, -10.8 (mV). D: PA63-NPs, -32.2 (mV)

and polydispersity index 0.340 (Fig 3A and B). Nanoparticles used for size distribution was investigated in the previous stages was used to prepare zeta potential.

Zeta potential of mPEG-PLA-NPs had an average peak in the range of -32.2 MV and PA63-NPs also have an average peak of -10.8 MV (Fig 3 C and D) (Table 2). In the research Arvind et al, the size of the nanoparticles produced from the PLA-PEG double-block copolymers was 219 to 239 nm [24].

Encapsulation efficiency and in vitro release profile of PA63 from NPs

The Encapsulation efficiency (%)and loading capacity of PA63 in NPs respectively decreased

Table 2. Characterization results of synthesized nanoparticles

Nanoparticles	Mean diameter (nm)	Encapsulation efficiency (%)	Zeta potential (mV)	Polydispersity Index
PA63-NPs	190	34.12	-32.2	0.340
mPEG-PLA-NPs	155	29.08	-10.8	0.034

Table 3. Encapsulation efficiency (%)and loading capacity of PA63-NPs against various addition volume of PA63

PA63 (mg)	Copolymer (mg)	Encapsulation efficiency (%)	Loading capacity (%)
1	100	34.12	0.352
2	100	29.08	0.603
3	100	21.46	0.664

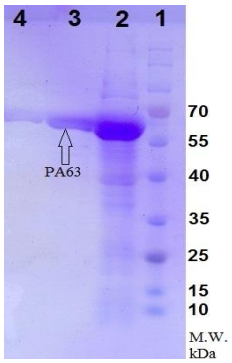

Fig 4. The quality of PA63 by SDS-PAGE analyze 12% stained with comassie blue. Lane 1: molecular mass standards. Lane 2: PA63 recombinant protein expressed in E.coli. Lane 3, 4: PA63 purified and before encapsulation (63 kDa), PA63 recovered after encapsulation

and increased when the amount of PA63 increasing (Table 3).

PA63 stability was investigated during the preparation of nanoparticles. After complete release of nanoparticles and comparison of released proteins with the recombinant protein as control, it was found that the recombinant protein did not get degraded during the nanoparticle manufacturing process and was stable (Fig 4).

The protein release profile of the NPs is shown in Fig 5. Only 26% of the total encapsulated protein was released on the first day and its release rate on days 7, 21, 42 was 33, 38, 54% respectively.

Evaluation of antibody titer in mice by indirect ELISA

Collected sera from each group analyzed for IgG titer using indirect ELISA. Based on the

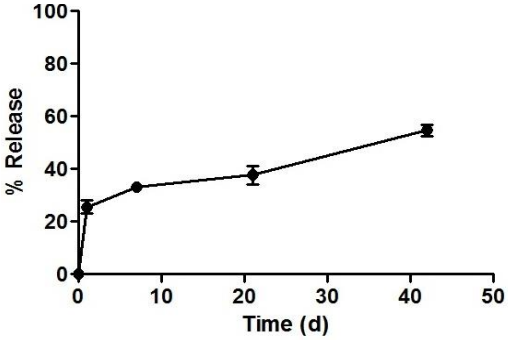

Fig 5. *In vitro* release profile of PA63 of the NPs

results, the highest IgG antibody titer in the PA63-NPs injection sample was the third blood sample that was taken one week after the last injection. Also in the first blood catch, the antibody titer was lower in the Nano formulation due to its slow release of recombinant protein (Fig 6).

The results show the ability of PA63-NPs to enhance the immune stimulation of the mice and increase the antibody titer compared to free PA63. Due to the results, injectable doses can be reduced, and the use of adjuvant is not required. Blank-NPs and adjuvant control did not show significant antibody titer.

Cell culture results

The results of cell culture assay at different concentrations of PA63-NPs 20, 40 and 60 µg/ml on Vero cells showed that the vaccine candidate had no significant cytotoxicity in any concentration of PA63-NPs in treated cells in comparison to control (P<0.05) (Fig 7).

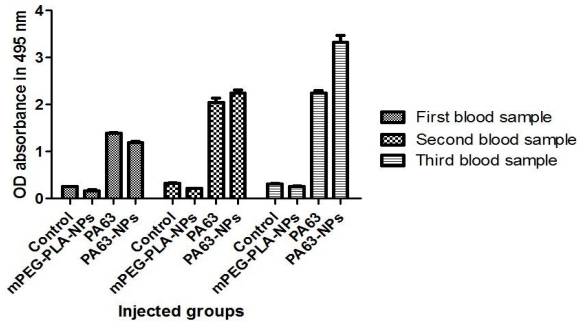

Fig 6. ELISA chart of IgG antibody production after injection of PA63-NPs, mPEG-PLA-NPs, PA63 and adjuvant control at dilution (1/200), OD absorbance in 495 nm. (P<0.05), animals (n= 5)

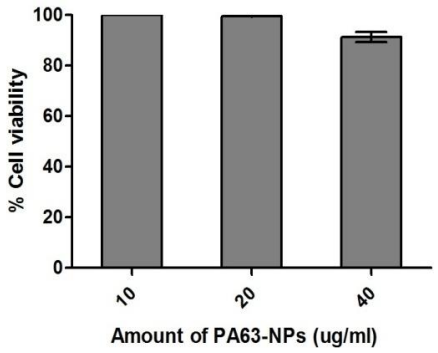

Fig 7. Cell viability of Vero cell with different concentration of PA63-NPs

DISCUSSION

PA is a very important component of anthrax toxin. This protein plays an important role in immunity against anthrax after immunization and during infection, and has an inhibitory effect on the LF and EF pathogenic factors [3, 25, 26]. Regarding the role of PA, it has been widely studied as a vaccine candidate [6-8]. The immunogenicity study of 2-4 domains and other PA domains in four different strains of mice was investigated and the expressed gene possessed immunogenicity and good adjuvant properties [27]. Typical PA vaccines show a very small protective response [28]. PA recombinant vaccines have not been able to solve the problem of multiple and annual doses [29]. Also, protein vaccines usually require adjuvant to increase the immune response. Aluminum hydroxide and phosphate salts are the agreed adjuvants in poison-based anthrax vaccines, but aluminum hydroxide exerts undesirable effects on PA in the long term and reduces its effect [29]. These adjuvants also have side effects and are sometimes toxic [22]. Therefore, the development of new formulations of anthrax vaccine can improve the quality of life of the patient and reduce the problems associated with repeated injections. Considering the mentioned reasons, in recent years, many studies have been carried out on the use of nanoparticles as vaccine carriers [30]. For example, delivery strategies such as polymeric Micro- and Nanosphere, biodegradable implants, injectable hydrogels and add PEG to carriers, have been investigated [32, 31]. The benefits of these drug delivery systems are the appropriate level of drug concentration in the blood for a long period of time, which prevents continuously drug injection [14, 16, 15]. Polylactic acid is one of a variety of biodegradable polymers that is widely used in vaccine delivery [24]. PLA has high hydrophobicity and too long decomposition time [33]. Various studies have shown that cited problems can resolve by copolymerization of PLA with hydrophilic polymers such as PEG [34]. Polyethylene glycol has good hydrophilicity, flexibility and biocompatibility and is resistant to detect by immune agents. These properties reduce the absorption of plasma proteins by forming a watery layer with having a low level of charge of PEG on the surface of the copolymer [17] and resulting increasing the stability of the nanoparticles in the body. Also research has shown that nanoparticles with a positive-charge are eliminated more quickly than nanoparticles with a negative superficial charge [34]. Vilaa et al, Used PLA-PEG double block polymers to prepare nanoparticles for protein delivery in the form of nasal and results showed that success of delivering the protein depends on the size of the produced nanoparticles, and the smaller particles make more success. Based on the results PLA-PEG double block nanoparticles are suitable carriers for protein delivery through the nasal route [35]. In a study conducted by Arvind et al, the polymerization of PEG and PLA was used to prepare a double block and three blocks copolymer for encapsulating HBsAg for mucosal vaccination against hepatitis B [24]. Polymeric vaccine delivery systems can release slowly and with a sustainable pattern. Controlled release has several benefits, including reducing dose changes, increasing efficacy, and minimizing side effects of the drug and vaccine, and tuning the drug in a range of suitable therapeutic concentration in plasma [23]. In this study the recombinant protein loading was carried out using water-oil-water emulsion method. First, the water-soluble recombinant protein was distributed in an organic solution and the water in oil emulsion was created. Subsequently, this complex was distributed in a water-solvent containing a surfactant and was created as water-oil-water emulsion. Generated nanoparticles require different checks to confirm. To investigate the size and zeta potential of produced nanoparticles by DLS, this non-destructive and fast method is used to determine the particle size in the range of several nanometers to microns [36]. DLS results in our current study showed that the loading of PA63 increased the surface potential of NPs. High value of zeta potential shows high surface charge of the nanoparticles, which directs to strong prevent interactions between nanoparticles and disperses them [37]. The results of the Zeta potential of nanoparticles produced showed that nanoparticles are within the range of stability. Also, the shape and size of nanoparticles produced by the SEM electron microscope showed that the produced nanoparticles are spherical and acceptable in shape and confirmed the size of the DLS results. After blood sampling, ELISA was performed with sera and the results showed that induces anti-PA IgG result of PA63-NPs was significantly higher compared to non-encapsulated PA63 proteins in third blood

sampling.

These results can be due to the slow release of recombinant proteins from nanoparticles, which makes the effective dose of the recombinant protein in the longer time available to the immune system of the mice and has a higher ability to stimulate the immune system. PEG in the structure of nanoparticles can also be a reason to increase the antibody titer. Because PEG coating can facilitate the access of nanoparticles to lymph nodes. Also recently reported that PEG can increase subcutaneous absorption [38]. The protein free nanoparticles (mPEG-PLA NPs) used as control had the lowest immune stimulation rate, indicating the high biocompatibility of the polymer used for the preparation of nanoparticles. The PLA decomposed by simple hydrolysis of the ester bond and produce hydroxyl-carboxylic acid and then metabolized to water and carbon dioxide citric acid cycle [33].

Cell culture results did not show any toxicity, which confirms the high results and confirms the biocompatibility and biosafety of our manufactured nanoparticles. The results of MTT assay showed that the encapsulated protein solution formulations that we used, do not have cytotoxicity comparison to control cells (P<0.05). According to above results, it can be stated that the Nano vaccine formulation can be proposed as a suitable carrier for the production of stable, safe, biocompatible vaccine and without the toxic effects of prevalent adjuvants.

In Manish et al research, PLGA polymer was used to encapsulate the PA domain 4 (PAD4). as a result, immunogenicity and titers of antibodies produced in Nano capsule form were observed compared to nonencapsulated state. Then, the protective effect of the Nano vaccine against spores of *Bacillus anthracis* was measured. The groups injected with PAD4-NPs survived for 6 days, and free protein groups survived for 1 day [21].

CONCLUSIONS

Based on the results of previous studies, the use of vaccine is the best way to fight anthrax.

Our purpose in this research is to prepare Nano formulation without adjuvant and stable recombinant anthrax vaccine. According to our research results, the mPEG-PLA double-block copolymer has a high ability to use as carriers to encapsulate the recombinant protein of the anthrax vaccine and improve the performance of this vaccine. It is suggested that quality control tests and approval of the required standards for human use be made regarding the candidate vaccine provided. It is also possible to industrialize the produce the recombinant protein production and its loading in mPEG-PLA double block copolymer.

In the future, hopefully access to vaccines would be more effective, safer and easier to use.

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