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Original Article

Evaluation of PLGA nanoparticles containing outer membrane proteins of *Acinetobacter baumannii* bacterium in stimulating the immune system in mice

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Abstract

Background and purpose: *Acinetobacter baumannii* (*A. baumannii*) is known as a pathogen with antibiotic resistance, causing respiratory infections. PLGA has been approved for use in vaccines as well as drug delivery. This study was performed to evaluate PLGA nanoparticles containing the outer membrane proteins (OMPs) of *A. baumannii* in stimulating the mice's immune system and improving pneumonia.
Experimental approach: Double emulsion solvent evaporation technique was used. The properties of the obtained nanospheres were determined using a zetasizer, FTIR, and AFM devices. Nanoparticles were administered to mice BALB/c by applying the intramuscular route. ELISA was used to measure the amounts of immunoglobulins produced; also, an opsonophagocytic killing assay was used to measure the effectiveness of immunoglobulins. Immunized mice were then challenged with live *A. baumannii* through the lungs; their internal organs were also removed for bacteriological studies.
Findings/Results: The prepared particles were 550 nm in diameter with a negative surface charge. The production of the OMPs specific IgG was much higher in the group receiving nanoparticles containing antigen as compared to those getting pure antigen. The immunoglobulins produced against nanoparticles were superior to those developed against pure antigens. Mice that received the new nanovaccine were more resistant to pneumonia caused by this bacterium than those that received pure antigen.
Conclusion and implication: Overall, it can be said that PLGA nanoparticles could deliver their internal antigens (OMPs) well to the immune system of mice and stimulate humoral immunity in these animals, thus protecting them against pneumonia caused by *A. baumannii*.

Keywords: *Acinetobacter baumannii*; Encapsulation; Nanoparticles; OMPs; PLGA.

INTRODUCTION

In recent years, infections caused by multidrug-resistant pathogens have become a serious problem, especially in the nosocomial setting (1,2). The World Health Organization (WHO) has recently identified microbial resistance as one of the three most important problems in front of human health. Some authors have summarized this phenomenon with the word 'ESKAPE', representing the most frequent multidrug-resistant microorganisms including *Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa* and *Enterobacter spp* (3).

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A. baumannii is an aerobic, polymorphic, and immobile gram-negative bacillus that is known as an opportunistic pathogen in immunocompromised individuals, especially those who have been hospitalized for a long time (4). Numerous studies have shown that the risk of *A. baummanii* infection is high in those patients who have used artificial devices such as catheters, sutures, and respiratory aids, or undergone dialysis or treatment with antibiotics for the past 90 days. The respiratory tract, blood, pleural fluid, urinary tract, surgical wounds, central nervous system, skin, and eyes may be places of infection (5,6). Pneumonia resulting from this bacterium may be a threat to the patient who needs mechanical ventilation. As *A. baumannii* can produce biofilms on the surface of the ventilator, it may have a relatively high level at the bottom of the respiratory tract (7-9). The use of vaccines as an effective and efficient therapeutic factor in the treatment of infectious diseases has been proven. To produce antibodies against small peptides and non-protein antigens such as saccharides and hapten molecules like most drugs, these molecules need to bind to a large immunogenic carrier. The use of nanoparticles as a new way to build carriers for colloidal vaccines has been investigated for immunization (10). Nanoparticles and microparticles are about the same size as viruses and bacteria that stimulate the immune system. Also, the size of nanoparticles is similar to cellular compounds entering the cell by different mechanisms (11). Poly (lactic-co-glycolic acid) (PLGA) micro and nanoparticles offer a promising future in the field of vaccine delivery systems. PLGA is a polymer ester of two alpha-hydroxy acids (lactic acid and glycolic acid). It has excellent properties. it has been approved by the US Food and Drug Administration for use in vaccines as well as drug delivery in tissue engineering due to its excellent human safety profile (12,13). PLGA particles are degradable and can be metabolized to their constituent monomers in aqueous media (14).

Outer membrane proteins (OMPs) are a group of unique intramembrane proteins located in the outer membrane of gram-negative bacteria and their β-plate structures are composed of 8 to 26 strands. OmpA (38 kDa) is the most abundant protein on the surface of this pathogen with immunogenic properties (15). Two reports based on immunoproteomics and reverse vaccination suggest that OmpA could have good potential for vaccine production against *A. baumannii* (16,17). The OMP 33 to 36 kDa (Omp 33-36), which acts as a water channel, is another outer membrane purine associated with the *A. baumannii* cytotoxicity (18). Omp22 is another outer membrane protein of *A. baumannii* that can be used in the production of vaccines to control the spread of infection by this bacterium. Both active and passive immunizations of Omp22 increased the survival rates of mice, suppressed the bacterial burdens in the organs and peripheral blood, and reduced the levels of serum inflammatory cytokines and chemokines (19). The aim of this study was comparing the immunogenicity of OMPs of *A. baumannii* in the pure and encapsulated form in PLGA nanoparticles as a nanovaccine in mice.

MATERIALS AND METHODS

Material and animals

The *A. baumannii* strain ATCC BAA-747 (PTCC 18555) was prepared by the Iranian Research Organization for Science and Technology. PLGA (lactide:glycolide 75:25; MW: 4-15 kDa, polyvinyl alcohol (MW: 85-124 kDa, 99% hydrolyzed), and horseradish peroxidase (HRP)-labelled anti-mouse IgG were all purchased from Sigma-Aldrich (St Louis, MO, USA). Female BALB/c mice (6-8 weeks of age weighing 17-20 g) were purchased from the Pasteur Institute of Iran (Karaj, Iran).

Bacterial culture

The bacteria were cultured in 500 mL of Müller-Hinton broth (MHB) medium and shaken at 37 °C for 5 days at 125 rpm. In order to kill the bacteria, 1 mL of Savlon was added. The bacterial masses were collected using a refrigerated centrifuge (Hettich-ROTIXA 50 RS, Germany) at 2500 rpm for 45 min; then stored at 4 °C.

Extraction of OMPs

Extraction of the OMPs of *A. baumannii* has been previously described by Cuenca et al. (20). Briefly, bacterial cells were washed twice with

10 mM phosphate buffer (pH 7.2) and then lysed on ice by sonication for 5-7 min in 30 s intervals (Hielscher UP100H, Germany). To separate the uncrushed cells, the mixture was centrifuged at 2500 rpm for 45 min; the supernatant was centrifuged at 13700 g for 45 min at 4 °C to pellet cell envelopes (MPW-350R). Cell envelopes were solubilized using 2% sodium lauroyl sarcosinate (Bio Basic Canada INC) in a10 mM phosphate buffer, at pH 7.2; this was for 30 min at room temperature. After solubilization, the insoluble outer membrane fraction was pelleted by centrifugation at 13700 g for 45 min at 4 °C. The outer membrane fraction was washed by resuspension in 2 mL 62.5 mM tris-HCl, pH 6.8, and centrifuged at 13700 g for 45 min at 4 °C. The obtained proteins were passed through cellulose acetate filters with 0.22 µm pores to be sterilized (MS® CA Syringe filters) and stored at -80 °C until use. The protein concentration was determined by the Bradford protein assay at the wavelength of 595 nm.

Profiling of the OMPs of A. baumannii

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to analyze proteins based on the differences in their molecular size. In this study, discontinuous SDS-PAGE was used to determine the protein profiles of *A. baumannii* using a previously described method by Shafiqul Islam *et al.* (21). Each lane of the gel was loaded with 15 µg of the OMPs. The proteins were separated on a 10% polyacrylamide gel by applying 25 mA for 40 min. The OMP obtained was resuspended with a sample buffer containing SDS and β-mercaptoethanol. After electrophoresis, the gel was stained with Coomassie brilliant blue R250 to see the desired bands. Protein standards were bovine serum albumin (BSA) (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa), and lysozyme (14.4kDa) (Bio-Rad Co., USA).

Fabrication of PLGA nanoparticles containing OMPs

Double emulsion solvent evaporation technique (water/oil/water) was used (13,22). To describe briefly, 2 mg of the OMPs was dissolved in 1 mL phosphate-buffered saline (PBS) as the inner water phase, and 50 mg of PLGA (lactide:glycolide 75:25; MW: 4-15 kDa) was dissolved in 2 mL of dichloromethane as the organic phase. A 5% (W/V) polyvinyl alcohol (MW: 85-124 kDa, 99% hydrolyzed) solution was then prepared as the external aqueous phase. OMPs aqueous solution (200 µL) was emulsified with 1 mL of organic phase using a probe ultrasonic processor (Hielscher Ultrasonic, Germany) with an ice bath for 1 min. The emulsion was stirred at 300 rpm, room temperature overnight. The nanoparticles were obtained by ultracentrifugation (14000 *g* for 30 min) (MPW 350R, Germany) and washed three times to remove excess polyvinyl alcohol and OMPs with deionized water. A volume of PBS equivalent to that used for OMPs was similarly encapsulated in PLGA to obtain PLGA-PBS. The nanoparticles were resuspended in double deionized water and lyophilized (SIM, FD8-8T, Newark, NJ, USA); they were stored at -20 °C until use.

Analysis of nanoparticles

To measure the zeta potential of the particles, 1 mg of OMP-PLGA nanoparticles were dissolved in 1 mL of deionized distilled water and sonicated in ice water for 15 min. Zeta potential was determined with a zeta sizer device (Malvern Nano ZS, UK).

An atomic force microscope (ARA-AFM) was then used to ensure the formation of nanoparticles and to determine their appearance. For this purpose, a drop of the formulation was poured on the relevant disk and was placed inside the microscope after drying. Images were taken in 5 × 5 and 20 × 20 µm dimensions.

Fourier-transform infrared spectroscopy (FTIR) is a method to obtain the absorption infrared spectrum of a substance, according to the absorption spectrum, its components and molecular structures. For this purpose, the desired formulation was poured into a filtered Amicon tube (Amicon® 10 kDa, Merck KGaA, Germany) and centrifuged at 5000 rpm. The top filter material was dried and ground with 0.1 g of potassium bromide, turning into tablets in a tablet maker. The tablet was inserted into the device (Bruker-Tensor 27, Germany) and the corresponding diagram was drawn.

Encapsulation efficiency

The direct method was used to determine the encapsulation efficiency percentage (EE%) (23,24). To describe briefly, 10 mg of PLGA nanoparticles containing OMPs was dissolved in 1 mL of dichloromethane and shaken at 37 °C for 30 min to evaporate the solvent. The dry matter was then dissolved in 1 mL of deionized water and centrifuged at 1000 g for 5 min to precipitate PLGA. Protein concentration in the supernatant was calculated by the Bradford protein assay. Each experiment was done in triplicate and the average was calculated. EE was then determined using the following equation:

$$\text{EE } \% = \frac{\text{Total } OMPs \text{ encapsulated}}{\text{Total } OMPs \text{ used for encapsulation}} \times 100$$

In vitro protein release

OMP-PLGA nanoparticles were suspended in normal saline using microtubes. The suspensions were incubated at 37 °C and the supernatants were collected by centrifugation at various time intervals (1, 2, 4, 6, 12, 18, 24, 36, 48 and 72 h). The released proteins in the supernatants were measured using the Bradford protein assay at the wavelength of 595 nm and replaced with the fresh buffer.

Immunization

A total of 60 female BALB/c mice ages 6 to 8 weeks, were divided into four groups; 15 mice each, kept in standard conditions at the relevant location for two weeks before the experiment. The animal study was in accordance with the guidelines of the laboratory animal administration Law of Iran, with the ethics No. IR.IAU.TNB.REC.1400.073, as approved by the ethics committee of Islamic Azad University, Tehran North Branch. The groups received three immunizations at two-week intervals on 0, 14, and 28 days with normal saline, PLGA-PBS, OMP, and OMP-PLGA *via* the intramuscular route. Mice in the OMP-PLGA group were immunized with 10 µg/500 µL of the encapsulated OMPs in normal saline; each mouse in the OMPs group was immunized with 7.72 µg of the nonencapsulated OMPs in 500 µL of normal saline; those in the PLGA-PBS group were immunized with an equivalent weight of PLGA-PBS nanoparticles. The control mice were immunized with normal saline only. Blood samples were taken from the hearts of the anesthetized mice two weeks after each vaccination period. For the better separation of sera, the blood samples were placed at 4 °C for 24 h; then the samples were centrifuged at 3000 rpm for 10 min. The collected sera were kept at -70 °C until the experiment.

Detection of antibodies

In the present study, an optimized ELISA was used to measure the serum levels of specific IgG responses. To describe briefly, 10 µg/mL of OMPs in the PBS buffer was prepared; to coat in the ELISA plate, 100 µL of antigens was dispersed into each well of 96-well ELISA plates (Greiner, Germany) and incubated overnight at 4 °C. Then the microplate was washed 3 times with a washing buffer. After that, 300 µL of the blocking buffer was added to each well and incubated for 1 h at room temperature. After rinsing with a washing buffer three times, 100 µL of the experimental diluted sera (serial dilutions from 1/2 to 1/256 in the dilution buffer) was poured into each well of 96-well plates. After incubating at room temperature for 2 h and washing 5 times, 100 µL of the 1:1000 dilution of the HRP-conjugated anti-mouse antibody (specific to mouse IgG; Sigma, USA) was added and the microplate was placed at 25 °C for 2 h. After rinsing the wells 5 times, 100 µL of tetramethyl benzidine solution was added to each of them and the plate was placed at 25 °C in the dark for 30 min. Finally, 50 µL of 2N H_2SO_4 as a stop solution was added to all wells and the absorbance of the wells at 450 nm was read by an ELISA reader device (STAT FAX 2100 - AWARENESS, USA).

Opsonophagocytosis assay

The opsonophagocytic killing assay was performed using the method developed by Paschall *et al.* though with some modifications (25). This method is an experimental procedure in which phagocytic cells are co-cultured with bacterial units. The immune cells will phagocytose and kill the bacterial cultures in a complement-dependent manner. First, to inhibit complement, the mice's serum was inactivated

at 56 °C for 30 min. The inactivated sera were serially diluted eight times (from 1/2 to 1/256) in a sterile 96-well culture microplate using the hanks buffer (10 µL/well). Ten µL of *A. baumannii* bacteria containing 1.5×10^5 colony-forming unit (CFU) was then added to each well and incubated at 37 °C for 30 min. As a complement source, baby rabbit serum was added to each well and the microplate was shaken at 150 rpm for 45 min at 37 °C. Thereafter, 40 µL/well (1×10^5 cells per well) of the mouse polymorphonuclears cells were added and incubated (90 min, at 37 °C, under agitation). Finally, NaCl 0.9% (80 µl/well) was added to each well and incubated (30 min, at 37 °C). One hundred µL of each well was plated on Müller-Hinton agar (MHA) and the plate was incubated for 24 h at 37 °C. The number of non-phagocytosed bacteria was then counted and calculated.

Experimental mice challenge

The pneumonia model previously described by Huang *et al*. was used with some minor modifications (26). Single colonies of *A. baumannii* which grew on MHA were used for inoculation. The bacteria were then cultured in the MHB at 37 °C / 200 rpm overnight. On the following day, the bacteria were subcultured by diluting 1:100 in fresh the MHB and cultured for 3 h at 37 °C / 200 rpm. The subculture was washed with PBS three times and adjusted to an optical density (OD_{600}), which was equal to 0.5. The inoculum was concentrated at 2×10^9 CFUs/mL and the experimental female BALB/c mice were infected with 50 µL of inoculum *via* aspiration. The inoculum CFUs were confirmed by plating on MHA plates and incubating them overnight at 37 °C. After three days of inoculation, the mice were anesthetized by diethyl ether, and their lungs, liver, and spleen were removed, weighed, and homogenized in 10 mL of sterile PBS; then dilutions of 0.1 and 0.01 were prepared. Subsequently, 1 mL of each was cultured in MHA and after 24 h, the colonies were counted.

Statistical analysis

To compare the results of the test groups, descriptive statistics methods and one-way ANOVA with the Tukey`s multiple comparison test using SPSS software, version 23 were used. *P* values < 0.05 were considered significant. All the experiments were repeated three times. The data were presented as mean ± SD.

RESULTS

SDS-PAGE of OMPs

The results of the SDS-PAGE of OMPs are shown in Fig. 1.

Zeta potential

The zeta potentials of PLGA nanoparticles and OMP-PLGA are shown in Fig. 2. The results showed that the surface charge of all nanoparticles had negative zeta potential values. The surface charge of PLGA nanoparticles was -21.5 mV; meanwhile, for the OMP-PLGA was -22.9 mV (Table 1).

AFM

The AFM images of OMP-PLGA and PLGA nanoparticles are shown in Fig. 3. By analyzing the images obtained from AFM with the Imager-ARA software, the morphology of the nanoparticles was determined. In order to compare the images better, their size was chosen to be exactly the same. The size of both nanoparticles was 550 nm (Table 1).

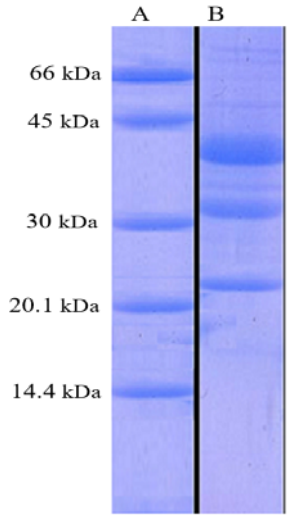

Fig. 1. SDS-PAEG gel showing the presence and location of the *Acinetobacter baumannii*'s OMPs. Lane A is the protein standards and lane B is the OMPs. OMPS, Outer membrane proteins.

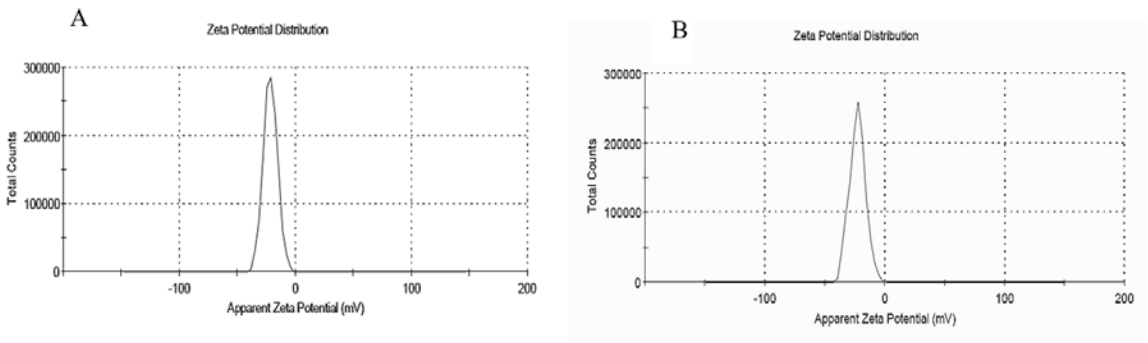

Fig. 2. Zeta potential charts of (A) PLGA and (B) outer membrane proteins-PLGA. PLGA, Poly (lactic-co-glycolic acid).

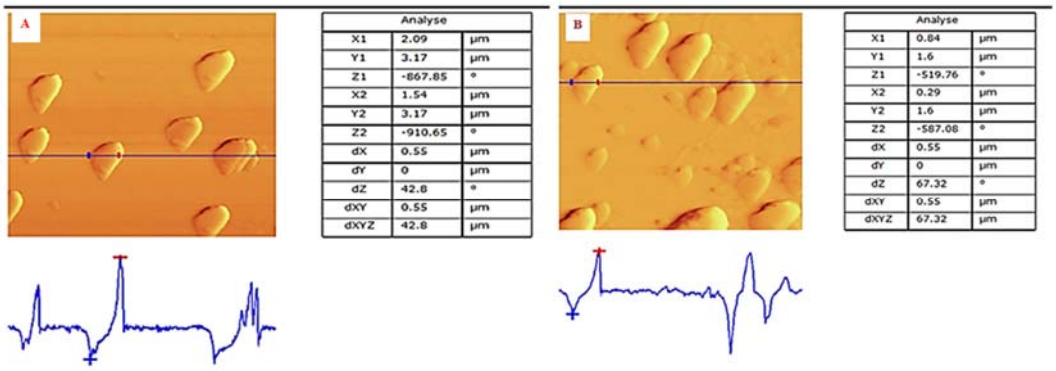

Fig. 3. Atomic force microscope images of (A) PLGA and (B) OMP-PLGA. OMP-PLGA, Outer membrane proteins-poly (lactic-co-glycolic acid).

Table 1. Structural characterization of OMP-PLGA nanoparticles.

Nanoparticles	Size (nm)	Polydispersity index	Zeta potential (mV)	*Encapsulation efficiency* (%)
PLGA	550	0.276 ± 6.017	-21.5 ± 6.14	-
OMP-PLGA	550	0.240 ± 0.014	-22.9 ± 6.72	77.2%

OMP-PLGA, Outer membrane proteins-poly (lactic-co-glycolic acid).

FTIR

The results of the FTIR spectrum of OMP-PLGA and PLGA nanoparticles are shown in Fig. 4. The presence of carbonyl, methyl and ether groups showed the structure of PLGA; the FTIR spectrum included the peaks 1700 cm^{-1}, 1400 cm^{-1}, and 1200 cm^{-1}, respectively. The presence of an amine group indicated OMPs in the spectrum of FTIR for the peak 3300 cm^{-1}.

There is an overlap in the two spectra FTIR of PLGA nanoparticles and OMP-PLGA in regions 1700 cm^{-1}(carbonyl), 1400 cm^{-1} (methyl), and 1200 cm^{-1}(ether) and the existence of the amine group in the region of 3300 cm^{-1} is a reason for the presence of protein in these nanoparticles.

EE and release studies of OMP-PLGA nanoparticles

The results showed that EE was approximately 77.2% (Table 1). The release of the OMPs from OMP-PLGA nanoparticles was measured by a sustained slow release over a 72 h period. The release of the OMPs from OMP-PLGA nanoparticles was 36.26%, 42.2%, 51.4%, 56%, and 56.3% of the total encapsulated proteins at 6 h, 12 h, 24 h, 48 h, and 72 h, respectively (Fig. 5).

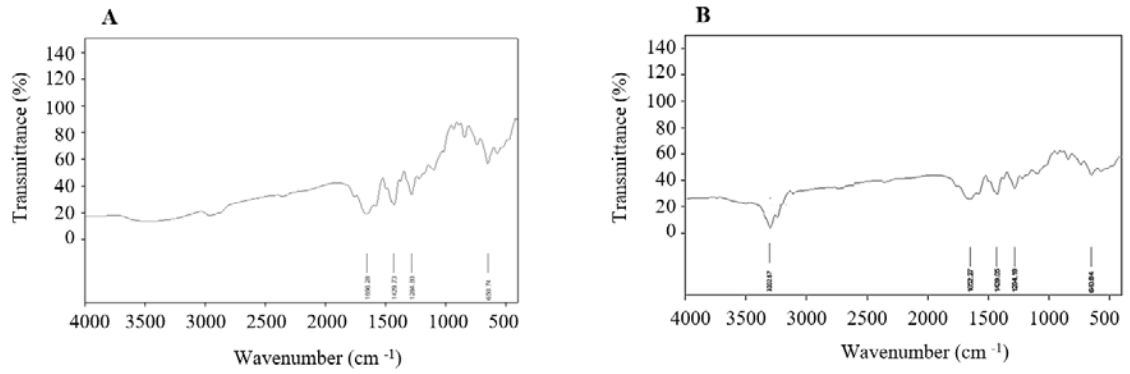

Fig. 4. The structural analysis results of (A) PLGA and (B) OMP-PLGA by FTIR analysis. OMP-PLGA, Outer membrane proteins-poly (lactic-co-glycolic acid).

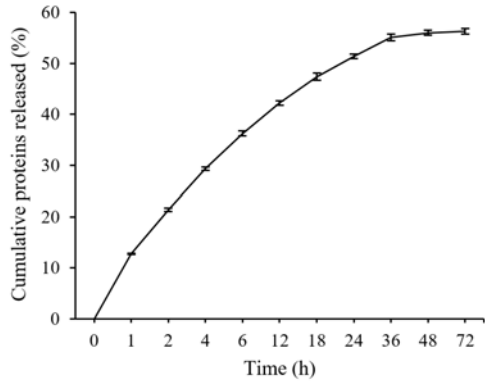

Fig. 5. *In vitro* release of OMPs from OMP-PLGA NPs. OMP-PLGA, Outer membrane proteins-poly (lactic-co-glycolic acid).

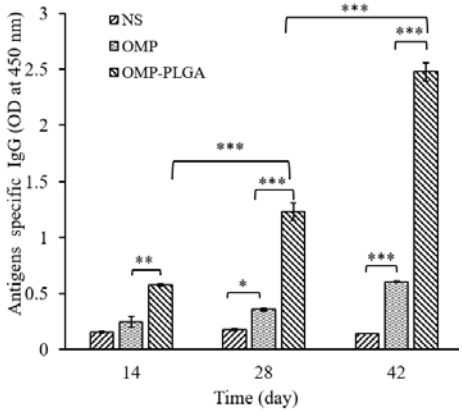

Fig. 6. Specific serum IgG titers against pure OMP and OMP-PLGA in three vaccination periods in the dilution 1/256: (mean level of antibody in 10 mice). **P* < 0.05, ***P* < 0.01, and ****P* < 0.001 indicate significant differences between groups. OMP-PLGA, Outer membrane proteins-poly (lactic-co-glycolic acid).

IgG response

The levels of antigen-specific IgG in the serum are shown in Fig. 6. The results showed that OMP-PLGA nanoparticles produced more antibodies when compared to pure OMPs. The serum IgG responses were increased after each vaccination period. The Immunoglobulin levels increased further after the second immunization period. The highest increase in the antibody response was in the third stage of immunization. In each immunization period, the groups immunized with the vaccines containing encapsulated antigen had more immune stimulation than the pure antigen (without PLGA). No increase in levels of antibodies was observed in both the control and PLGA recipient groups during the experimental period.

Opsonophagocytosis studies

The details of the opsonophagocytic killing are shown in Fig. 7. In all serum dilutions, serum opsonophagocytosis was superior in the group that received OMP-PLGA, rather than the one receiving OMPs alone. The highest rate of opsonophagocytosis was observed in dilution 1. The rate of opsonophagocytosis was decreased linearly in the serum dilutions. The results of opsonophagocytosis calculations for one of the dilutions (1:32) are shown in Table 2. Wells containing only bacteria were used as CFU basis points and those without serum after incubation were considered as a reference for calculating the CFU reduction.

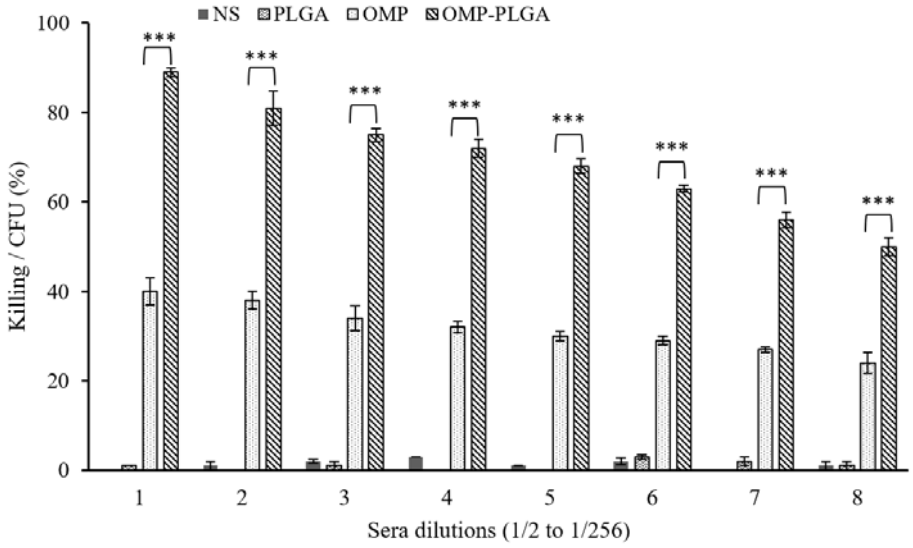

Fig. 7. The percent of opsonophagocytosis activity in various serum dilutions of experimental mice that were immunized with OMP and OMP-PLGA. There was a significant difference between the two groups in all serum dilutions but significant differences in % inhibition of opsonophagocytic killing activity between NS and PLGA recipient groups were not significant. ****P* < 0.001 Indicates significant differences between defined groups. OMP-PLGA, Outer membrane proteins-poly (lactic-co-glycolic acid), NS, normal saline.

Table 2. *Comparison of the opsonophagocytosis activity of Acinetobacter baumannii in the 1:32 serum dilution.*

Antigen injection (n = 10)	**Opsonophagocytic activity Mean unit numbers CFU/well**	**Inhibition of opsonophagocytic killing activity (%)**	***P* values**
OMP	407 ± 15	30%	< 0.001
OMP-PLGA	174 ± 21	68%	< 0.001
PLGA	530 ± 16	-	-
Normal saline (negative control)	545 ± 27	-	-

OMP-PLGA, Outer membrane proteins-poly (lactic-co-glycolic acid).

Protection studies

The results of the mice challenged by live *A. baumannii* showed that there was a significant difference in the number of bacteria in the lung, spleen, and liver of immunized mice as compared to the control ones. Furthermore, there was a significant difference between the two groups of pure OMPs and OMP-PLGA recipients in all three culture samples (in dilution 0.01; Fig. 8). The best level of protection in the OMP-PLGA group was for the lungs. However, there was no protection for the control group and the PLGA-receiving group against the *A. baumannii* strain ATCC BAA-747.

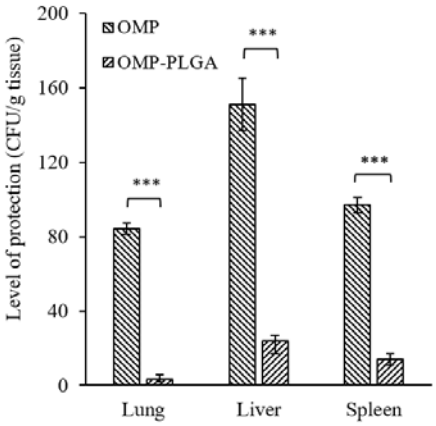

Fig. 8. Level of protection against *Acinetobacter baumannii* infection in two groups receiving OMP-PLGA and OMP in the 1:100 dilution. ****P* < 0.001 Indicates significant differences. OMP-PLGA, Outer membrane proteins-poly (lactic-co-glycolic acid).

DISCUSSION

A. baumannii has recently emerged as one of the most important health problems due to its propensity to acquire multidrug, extensive drug and even pandrug-resistance phenotypes at rates not previously seen (3-9). New ways to prevent and treat such infections are of critical medical need. Despite decades of effort to develop A. *baumannii* vaccines, there is still no effective vaccine against this pathogen. Recently, many attempts have been made to develop a vaccine using the virulence agents of *A. baumannii*; pure protein-based vaccines are one of them (27). Previous studies have identified OMPs of this bacterium (16,17) and investigated the immunogenicity of some of them in mice (15,18,19).

The use of PLGA nanoparticles in vaccine delivery systems and immune stimulants in different cases has been previously studied (28). Encapsulation of protein antigens inside PLGA nanoparticles is one of the methods that has been used in various cases before (13). For example, in one study, hepatitis B virus surface antigen (HBsAg) was loaded into PLGA particles showing increased immune stimulation by controlling the antigen release (29). In another study, mice immunized with the recombinant major outer membrane protein of *Chlamydia trachomatis* encapsulated in PLGA nanoparticles produced a higher serum IgG in comparison to those immunized with recombinant major outer membrane protein in the Freund's adjuvant (30). One other study showed that the recombinant OMP-22 of *A. baumannii* encapsulated in chitosan-PLGA elicited specific IgG antibodies, Th1 cellular immunity and protection against acute lethal intratracheal *A. baumannii* challenge (31). In this study, the immunogenicity of the encapsulated OMPs of *A. baummani* in the PLGA nanoparticles and purified OMPs were evaluated in the BALB/c mice model.

Comparison of the profiles of *A. baumannii* OMPs using SDS-PAGE with the previous study (21) showed that the desired proteins were well extracted.

The morphological characteristics of nanoparticles can affect the release of biomaterials from them. A porous structure enhances the release of biomaterials by diffusion, whereas a smooth surface reduces the burst release (14). In the present study, the physical and structural characteristics of OMP-PLGA revealed that it had a homogeneous morphology with a smooth spherical shape that was also uniform in particle size distribution and fairly dispersed without aggregation (Fig. 3).

Another concern regarding nanoparticle formulations is the value of the zeta potential, which is important for understanding and predicting the long-term stability and adhesion of nanoparticles; in theory, the more negative or positive zeta potential, the more stable the nanoparticle suspension (30). According to other studies, PLGA normally produces spherical nanoparticles with a negative charge (13). Our results showed that OMP-PLGA was stable and had a negative surface charge of -22.9 mV, which could be attributed to the addition of polyvinyl alcohol to the suspension. It should also be noted that the amount of the potential at the surface of PLGA particles varied at different pHs (32).

Encapsulation efficiency is one of the factors indicating the amount of the desired antigen which is embedded in nanoparticles. Different values have been reported in different studies. For example, in one study of hepatitis B virus surface antigen, this value was about 76% (29). In another study, the encapsulation rate of tetanus toxoid within PLGA particles was 69% (33). In this study, encapsulation efficiency was 77.2%, which is acceptable.

The release pattern of proteins in nanoparticles can influence the immune response, the proteins concentration and the frequency of immunization. Makadia and Siegel described drug release through PLGA particle degradation as a biphasic curve with an initial burst release (first phase) followed by progressively increasing drug release (second phase) (14). In the present study, OMP-PLGA diffusion profiles followed this pattern. The burst release on the first day could induce a strong immune response. The sustained slow release of the proteins was an attractive property for a vaccine candidate as this might reduce the number of immunizations, as well as enhancing the presentation of the proteins to antigen-presenting cells.

Surprisingly, according to the results of different studies, the effect of particle size on eliciting immune response has been inconsistent. The size of nanoparticles is relatively similar to that of pathogens easily detected and eliminated by the immune system. In general, it can be said that PLGA particles with a size between 200 nm and 2 µm can induce better immunity responses (13). In the present study, the particle size was 550 nm which was appropriate for stimulating the mice's immune system.

The results of one study showed that the nasal route, with cholera toxin as a mucosal adjuvant, was successful and could be used to induce sera IgG antibodies against the purified OmpA of *A. baumannii* (15). Another study also showed that the specific IgG response was induced efficiently by the purified recombinant Omp22 of *A. bumannii* in mice immunized subcutaneously by different doses of Trx-Omp22 proteins, with 1 mg alum as the adjuvant (19). Further, the results of a new study showed that chitosan-PLGA-rOmp22 immunized BALB/c mice were induced by higher levels of rOmp22-specific IgG in serum when they were immunized subcutaneously (31). In this study, it was found that exposing mice to the OMPs of *A. baumannii* from the intramuscular injection route also induced the production of serum IgG. As shown in Fig. 5, both OMP and OMP-PLGA intramuscular injections induced a humoral response. However, high titers of antigen-specific IgG antibodies were detected in the serum of OMP-PLGA-immunized mice after each immunization period, as compared with the OMP receiving group (especially after the second boost), thus showing a significant difference between them ($P < 0.001$). These results also indicated that PLGA nanospheres as an adjuvant could introduce their internal antigens (OMPs of *A.baumannii*) into the mice′s immune system, thus inducing more humoral immunity into the pure antigen.

The opsonophagocytic killing assay is used as a correlate for protection to measure the functional capacities of vaccine-candidate-raised antibodies. A previous study showed that Omp-22-produced immunoglobulins in mice increased the opsonophagocytic activity in a complement-dependent manner (19). The results of this study were similar, showing that immunoglobulins produced against *A. baumannii*'s OMPs had the most ability to opsonize live bacteria to mice polymorphonuclears cells. In the group receiving OMP-PLGA, the increase in immunoglobulin efficiency was 38% higher than that in the one getting OMP (in the 1:32 dilution). Also, in all dilutions, the results of the immunoglobulin analysis for the two groups receiving antigen (OMP and OMP-PLGA) showed that the highest rate of opsonization was produced by immunoglobulins of the OMP-PLGA group; there was also a significant difference between the two groups in all serum dilutions ($P < 0.001$). Previous studies have also shown that mice immunized with OMPs of *A. baumannii* were protected against live pathogenic bacteria (15,18,19). The results of another study showed that mice immunized with chitosan-PLGA-rOmp22 were protected against infection with *A. baumannii* ATCC 19606 and three clinical strains of *A. baumannii*; the bacterial load in the blood of the mice inoculated with chitosan-PLGA-rOmp22 after infection was much lower than that of the nonencapsulated rOmp22 group and the control groups (31). The results of the challenge test in this study showed that the reduction of the microbial burden in the internal organs of mice (lung, liver, and spleen) and protection rates in mice immunized with OMP-PLGA were much more than those in the group receiving OMP alone and there was a significant difference between them in all three culture samples (in dilution 1/100, $P < 0.001$). These results also indicated that the high titer of antigen-specific antibodies contributed to potent protection in the mice immunized with OMP-PLGA.

CONCLUSION

The double emulsion solvent evaporation technique (water/oil/water) was found to be suitable for the preparation of microspheres containing OMPs of *A. baumannii*. Particle size was also appropriate for stimulating the mice's immune system. OMP-PLGA nanoparticles elicited specific IgG and the produced immunoglobulins had high efficiency to

increase opsonization activity. There was protection against the acute lethal intratracheal *A. baumannii* challenge in BALB/c mice. Generally, OMP-PLGA nanospheres can immunize against *A. baumannii* ATCC BAA-747, thus serving as the basis for the production of nanovaccines against this bacterium.

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Conflict of interest statement

The authors declared no conflict of interest in this study.

Authors' contributions

R. Shapoury proposed the experiments and research design and analyzed the results of solid-state characterizations; A. Gholizadeh performed the experiments under the supervision of R. Shapoury and P. Pakzad, and wrote the manuscript; P. Pakzad supervised immunological and animal experiments; M. Mahdavi analyzed the results of immunology study and helped to write the manuscript; H. Danafar helped to perform the experiments and analyzed the results of drug study. The final version of the manuscript has been approved by all authors.

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