A Multi-epitope Peptide rOmp22 Encapsulated in Chitosan-PLGA Nanoparticles as Vaccine Candidate Against *Acinetobacter baumannii* Infection

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Research

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Abstract

Background: Development of vaccine is a promising and cost-effective strategy to prevent emerging multi-drug resistant (MDR) *Acinetobacter baumannii* infections. The purpose of this study was to prepare a multi-epitope peptide nanovaccine and evaluate its immunogenicity and protective effect in BALB/c mice.

Results: The B-cell and T-cell epitopes of Omp22 from *A. baumannii* were predicted using bioinformatics method and identified by immunological experiments. Three dominant B-cell epitopes and two T-cell epitopes were linked in series and chemically synthesized to generate multi-epitope peptide rOmp22. Then, rOmp22 was encapsulated by chitosan (CS) and polylactic acid glycolic acid (PLGA) to prepare CS-PLGA-rOmp22 nanoparticles (NPs). CS-PLGA-rOmp22 NPs were small (mean size of 272.83 nm) with apparently spherical structural, positively charged (4.39 mV) and exhibited nontoxicity to A549 cells. We achieved a high encapsulation efficiency (54.94%) and a continuous slow release pattern. Compared with non-encapsulated rOmp22, CS-PLGA-rOmp22 induced more rOmp22-specific IgG in serum and IFN- γ in splenocyte supernatant. Vaccination with CS-PLGA-rOmp22 decreased lung injury, suppressed bacterial burdens in the lung and blood, provided potent protection (57.14%-83.3%) against acute lethal intratracheal *A. baumannii* challenge in BALB/c mice.

Conclusions: CS-PLGA-rOmp22 NPs could elicit specific IgG antibody, Th1 cellular immunity and protection against acute lethal intratracheal *A. baumannii* challenge. Our results indicate this nanovaccine is a desirable candidate to prevent *A. baumannii* infection.

Background

Acinetobacter baumannii is an opportunistic pathogen that predominantly causes pneumonia, bacteremia, meningitis, and urinary tract infections in patients. *A. baumannii* has recently emerged as one of the most important health problems due to its propensity to acquire multi-drug, extensive drug and even pan-drug resistance phenotypes at previously unforeseen rates[1–3]. The main antibiotic resistant mechanisms include alteration in the target sites, failure in the degradation-specific enzymes, perfusion defects and modification in multidrug effusion pumps[1, 4]. Besides, *A. baumannii* has a number of potential virulence factors, such as siderophore-mediated iron-acquisition system and biofilm formation, which could possibly affect clinical outcomes[5]. Global emergence of multi-drug resistant (MDR) and pan-drug resistant (PDR) *A. baumannii* have resulted in significantly increased mortality rates with limited or no options for therapeutic interventions[6, 7].

Vaccination strategies are emerging as a viable option to prevent or treat MDR or PDR infections, but there is still no licensed vaccine against *A. baumannii*. Conventional vaccines developed from live attenuated or inactivated whole cells could induce strong humoral and cellular immunity, however, the clinical applications of such vaccines have been limited due to their complex compositions and potential safety concerns[8–10]. During the last decades, the research of vaccines against *A. baumannii* has

primarily focused on various forms of recombinant antigens, including biofilm-associated protein Bap[11], auto-transporter (Ata)[12], outer membrane protein A (OmpA)[13], outer membrane protein assembly factor (BamA)[14], Poly-N-acetyl- β -(1–6)-glucosamine (PNAG)[15], and outer membrane protein 22 (Omp22)[16]. Animal studies showed that some single recombinant protein based vaccines provided only weak protection against *A. baumannii* infection or poor cross-protection against certain strains[17]. In addition, the formulation of such vaccines often leads to reactogenic and/or allergenic responses that are often not desired[18]. Thus, determining an antigen that has high immunogenicity and avoids the virulence of structural proteins is the key to prepare an *A. baumannii* vaccine.

Recently, the design of epitope-driven or peptide-based vaccines is becoming more attractive, because they are comparatively easier to produce and construct, lack any infectious potential and offer chemical stability[18–20]. There are many multi-epitope vaccine design studies involving various bacteria like *Klebsiella pneumoniae*[21], *Shigella sonnei*[19] and Meningitis-inducing Bacteria (*Streptococcus pneumoniae*, *Neisseria meningitidis*, and *Haemophilus influenzae* Type b)[22]. Ren et al[23] firstly designed a multi-epitope assembly peptide (MEP) of *A. baumannii* and evaluated its immunogenicity and protective immunity in BALB/c mice. The results of that study indicated that the rMEP is a promising vaccine candidate for the control of infections caused by *A. baumannii*. However, to be optimally effective, peptide-based vaccines need to be administered with adjuvants. Many currently available adjuvants are toxic, not biodegradable and invariably invoke adverse reactions, including allergic responses and excessive inflammation. A nontoxic, biodegradable, biocompatible vaccine delivery system is urgently needed.

The nanotechnology-based approach is considered to be one of the most advantageous methods for the development of peptide-based vaccine[24]. Poly (lactic-co-glycolic) acid (PLGA) is a synthetic copolymer that has been approved by United States Food and Drug Administration (FDA) and European Medicine Agency (EMA) for various medical and pharmaceutical applications in humans[25]. PLGA NPs have been shown to be efficient for antigen delivery because of their effectiveness in enhancing immune responses, controlled release, high encapsulation efficiency and tissue bio-distribution, along with being biodegradable, non-toxic and small in size[26–28]. While PLGA NPs display many advantages in antigen delivery, in comparison with cationic bio/polymers, they can suffer poor encapsulation efficiency and instable when loading negatively charged molecules, such as protein or peptide antigen. Chitosan (CS) is a non-toxic and non-immunogenic naturally occurring linear amino poly-saccharide (poly 1,4-day-glucoamine), with an ability to enhance the penetration of large molecules across mucosal surfaces[29]. Such anionic PLGA NPs can be subjected to chitosan-surface coating, thus resulting in cationic chitosan-coated PLGA (CS-PLGA) NPs, which hold promise as innovative formulations for targeted delivery[27]. Previous studies have demonstrated that CS-PLGA NPs are particularly effective for antigen delivery to APC, such as inducing antigen presentation to lymphocytes or modulating APC function[27].

In this study, we chose *A. baumannii* outer membrane protein 22 (Omp22), a highly conserved and highly immunogenic protein, as the candidate antigen. Previous study has found that immunization with recombinant Omp22 efficiently elicited high titers of specific IgG, increased the survival rates of mice, and

suppressed the bacterial burdens in the organs and peripheral blood[16]. However, Omp22 is not only a key protein involved in *A. baumannii* metabolic process, but also has certain toxicity[16]. Therefore, we used bioinformatics techniques and immunological methods to predict and identify optimal T-cell and B-cell epitopes on *A. baumannii* Omp22 protein. Subsequently, the identified dominant epitopes were connected in series by 6-aminocaproic acid and chemically synthesized to generate multi-epitope peptide rOmp22. Then, rOmp22 was encapsulated by CS-PLGA to prepare multi-epitope peptide nanovaccine (CS-PLGA-rOmp22). The physical-structural characterization, immunogenicity and protection efficacy of the vaccine were evaluated comprehensively *in vitro* and *in vivo*. This novel nanovaccine can retain the corresponding immunogenicity of Omp22, and avoid its harmfulness to the host, which should become a priority strategy against *A. baumannii* infection.

Results

Heterologous expression and purification of recombinant Omp22

The recombinant Omp22 protein was synthesized and purified for further study. The Omp22-encoding gene was amplified by PCR, cloned into the plasmid pET-28a (+), and transformed into *Escherichia coli* (*E. coli*) BL21 (DE3). The positive clones were confirmed by restriction (Fig. 1a) and sequencing (Additional file 1: Fig. S1). The recombinant protein Omp22 was expressed in *E. coli* BL21 (DE3) and was purified with Ni-IDA resin. The purified protein was analyzed by SDS-PAGE, a specific band was seen at 24 kDa, which was consistent with the expected size (Fig. 1b).

Epitope prediction and design of multi-epitope peptide rOmp22

According to bioinformatics analysis, such as secondary structure of epitope, surface accessibility, hydrophilicity, flexibility and antigen index, four candidate B-cell epitope peptides (named Omp22 B1-Omp22 B4) and four candidate T-cell epitope peptides (named Omp22 T1-Omp22 T4) (Table 1) were predicted and chemically synthesized.

Table 1
Location and amino acid sequence of candidate antigen epitopes of Omp22 protein

Epitopes	Location	Amino acid sequence	Epitopes	Location	Amino acid sequence
Omp22 B1	175–182	GKGVPSSR	Omp22 T1	112–123	TFDTNKSNIKP
Omp22 B2	158–172	NIPLSQARAQSVKNY	Omp22 T2	153–164	GNDSINIPLSQ
Omp22 B3	125–135	YATLDKVAQTL	Omp22 T3	178–189	VPSSRIDAQGY
Omp22 B4	102–108	SVQLIMP	Omp22 T4	204–215	EQNRRVEISIIY
Note: location indicate the position of amino sequence in Omp22 protein.					

BALB/c mice were subcutaneously injected with 100 µg recombinant Omp22 (1 µg/µl in PBS) or an equal volume of PBS for three times with two-week interval. One week after the last immunization, blood and spleen were collected for further immunology study, the schedule of vaccination are shown in Fig. 1c. Three B-cell epitopes (amino sequences: NIPLSQARAQSVKNY; YATLDKVAQTL-SVQLIMP) were recognized by serum from mice immunized with Omp22 whole-length protein (Fig. 1d). Splenocytes from Omp22 protein immunized mice could be stimulated by two synthetic T-cell epitopes (amino sequences: VPSSRIDAQGY; TFDTNKSNIKP) (Fig. 1e). The identified optimal T-cell epitopes and B-cell epitopes were connected in series by 6-aminocaproic acid to design and generate a novel multi-epitope peptide (MEP) rOmp22 (Fig. 1f). MEP rOmp22 with 59 amino acid and molecular weight of 6535.48 Da was chemically synthesized and identified by liquid chromatography and mass spectrometry (Additional file 1: Fig. S2).

Physical-structural characterization of nanoparticles

The characteristics of CS-PLGA-PBS and CS-PLGA-rOmp22 nanoparticles were summarized in Table 2. Transmission electron microscopy (TEM) techniques were employed to assess the morphology and size of the nanoparticles. By TEM analysis, CS-PLGA-PBS (Fig. 2a) and CS-PLGA-rOmp22 (Fig. 2b) NPs appeared to be apparently smooth with evenly double spherical structure. Particle size analysis showed that the diameter of CS-PLGA-PBS and CS-PLGA-rOmp22 NPs were at 295.78 ± 21.28 nm (Fig. 2c) and 272.83 ± 15.57 nm respectively (Fig. 2d), with nano-range size (100–300 nm) and moderate uniformity (PDI around 22.8%). As shown in Fig. 2e and 2f, the absolute value of the zeta potential for CS-PLGA-PBS and CS-PLGA-rOmp22 were about 3.70 and 4.39 mV, indicating a high repulsion between the nanoparticles, which kept the NPs in a state of dispersion instead of aggregation or clumping.

Encapsulation efficiency and release studies of CS-PLGA-rOmp22 nanoparticles

We calculated the encapsulation efficiency (EE) by high performance liquid chromatography (HPLC) method. The results showed that EE was about 55% and loading capacity was about 0.94% (Table 2). The release of the rOmp22 peptide from CS-PLGA-rOmp22 NPs was measured by a sustained slow release over a 72 hours period. The release of rOmp22 peptide from CS-PLGA-rOmp22 was found to be 26.2%, 40.6%, 51.5% and 60% of the total encapsulated peptide on 12 h, 24 h, 48 h and 72 h, respectively (Fig. 3a).

Table 2
Nanoparticle size distribution, Zeta potential, and encapsulation efficiency

Nanoparticles	Size (nm)	PDI (%)	Zeta potential (mV)	EE (%)	LC (%)
CS-PLGA-PBS	295.78 ± 21.28	22.83 ± 1.68	3.70 ± 1.07	/	/
CS-PLGA-rOmp22	272.83 ± 15.57	22.85 ± 1.76	4.39 ± 0.69	54.94	0.94
Note: Values are shown as the mean ± SD for the size, PDI and zeta potential.					
Abbreviations: PLGA, poly D, L-lactide-co-glycolide; PBS, phosphate-buffered saline; PDI, Polydispersity index; EE, encapsulation efficiency; LC, loading capacity.					

Cytotoxicity effect of rOmp22 and CS-PLGA nanoparticles on A549 cells

Toxicity is of major concerns when using nanoparticles, even biodegradable polymers, in biomedical applications. We tested the toxicity of rOmp22 and nanoparticles against human lung adenocarcinoma epithelial cell line A549. Both dose- and time-dependent toxicity studies were conducted by A549 cells exposing to rOmp22 ranging from 1.25 µg/ml to 80 µg/ml and CS-PLGA nanoparticles ranging from 12.5 µg/ml to 800 µg/ml over a 6-, 24- and 48-h time period. At all examined time points, all concentrations of rOmp22 (Fig. 3b) and nanoparticles (Fig. 3c, Fig. 3d) did not affect the viability of A549 cells, which demonstrated that they were non-toxic to A549 cells, and substantiated their safety and biocompatibility for studies *in vivo*.

Generation of antibody-mediated immune responses

To measure the antibody-mediated immune responses at different vaccination regimens, BALB/c mice were subcutaneously injected with 40 µg rOmp22 or CS-PLGA-rOmp22 nanovaccine containing equal rOmp22. Mice injected with CS-PLGA-PBS or adjuvant were as control groups. Serum samples were collected from immunized mice after the third immunization (Fig. 4a). We investigated the capacity of encapsulated rOmp22 to potentiate antibody immune responses in mice by quantifying rOmp22-specific antibodies using ELISA. As shown in Fig. 4b, immunization with CS-PLGA-rOmp22 elicited significantly ($P < 0.01$) higher IgG compared to rOmp22 immunized mice. No antigen-specific antibody was detected in the serum from the CS-PLGA-PBS control group or the adjuvant-treated group.

To further define the robustness of the rOmp22 specific antibody, we next performed serial two-fold dilutions of sera to determine rOmp22 specific antibody titers. As shown in Fig. 4c and Fig. 4d, sera from the CS-PLGA-rOmp22 mice had higher antibody titers and endpoint titers in comparison with those from non-encapsulated rOmp22 immunized mice and the control mice.

Splenocyte stimulation and measurements of cytokines IL-4 and IFN-γ

Splenocytes were isolated from six mice in each group at day 7 after the third immunization and cultured with the stimulation of rOmp22. IL-4 and IFN- γ secretion in the culture supernatants were detected by ELISA method. The splenocytes from the CS-PLGA-rOmp22 immunized mice produced higher levels of IFN- γ than those from rOmp22-immunized group ($P < 0.01$) (Fig. 4e), whereas IL-4 secretion showed no difference between CS-PLGA-rOmp22 group and non-encapsulated rOmp22 group (Fig. 4f).

Flow cytometry analysis

Seven days after the last immunization, cells were harvested from the spleen and the draining lymph node. Flow cytometry analysis showed that nanovaccine CS-PLGA-rOmp22 induced significantly more CD 19⁺ B cells and less CD 3⁺ T cells in the lymph node after immunization compared with non-encapsulated rOmp22 (Fig. 5a and 5c). There was no difference of CD 19⁺ cell levels in spleen among mice in different groups (Fig. 5b). The frequencies of CD 3⁺ cells were somewhat increased in the spleen of CS-PLGA-rOmp22 immunized group (Fig. 5d). No significant differences were observed in the frequencies of CD 11c⁺ dendritic cells (DC) and CD 11b⁺ CD 169⁺ macrophages (MAC) in spleen and draining lymph node (Fig. 5e-h). The CS-PLGA nanovaccine increased the expression of major histocompatibility complex class II (MHCII) on CD 11c⁺ DC by twofold in the draining lymph node compared to unencapsulated rOmp22 peptide (Fig. 5i), while no significant difference was observed in the spleen across any group (Fig. 5j). The expression of MHC II marker on CD 11b⁺ CD 169⁺ MAC in spleen and lymph node increased in the CS-PLGA-rOmp22 administrated mice, though there was no significant difference compared with non-encapsulated rOmp22 group (Fig. 5k and 5 l).

Immunizations reduced bacterial loads

Antibody and cell-mediated immunity are just predictors for immune responses. To determine whether CS-PLGA-rOmp22 vaccine conferred protection, we assessed its effectiveness using an acute pneumonia model in BALB/c mice. Two weeks after the last boost immunization, all groups were challenged with lethal dose of *A. baumannii* ATCC19606 (2×10^8 CFU/mouse) and three clinical *A. baumannii* strains, CS-MDR-AB (1×10^9 CFU/mouse), CRAB (5×10^8 CFU/mouse) and PDR-AB (5×10^8 CFU/mouse) via the trachea. Drug sensitive test results of four *A. baumannii* strains were shown in Additional file 1 (Table S1). Six mice were randomly selected from each group for the detection of bacterial load in the blood and lung tissue (Fig. 6a).

Blood samples collected from six mice in each group in 24 h post-challenge were serially diluted and plated on LB agar plates followed by incubation at 37°C overnight. The number of CFUs was counted, and the log₁₀ CFU/ml was calculated and compared. Mice immunized with CS-PLGA-rOmp22 had lower bacterial loads in the blood than mice from the other three groups (Fig. 6b and 6c).

After taken blood, six mice in each group were sacrificed, lungs were collected. The right lung lobes were prepared for CFU assessment. As shown in Fig. 6d-g, the lungs from CS-PLGA-rOmp22 groups showed significantly lower bacterial burden than those two control groups. Further, the bacterial burden was significantly reduced in the CS-PLGA-rOmp22 vaccinated group compared with the non-encapsulated

rOmp22 group. These results indicated that immunization with CS-PLGA-rOmp22 could partially reduce the colonization of *A. baumannii* in the lung of mice.

Pathological changes in mouse lung tissue

The left lung tissue was aseptically collected in 24 hours post-challenge and fixed in 4% buffered formalin, stained with hematoxylin-eosin and observed under microscope. As shown in Fig. 7, the lung slices of the CS-PLGA-rOmp22 group showed less extensive of lung lesions, alveolar edema, lymphocyte infiltration and structural damage caused by inflammatory response compared with the rOmp22 vaccine group, CS-PLGA-PBS group and adjuvant control group. Moreover, in the CS-PLGA-rOmp22 vaccine groups, there were more normal structure and clearer alveoli than other three groups. The results of severity score showed that the severity of lung injury in CS-PLGA-rOmp22 groups was significantly lower than the other groups (Fig. 7c-f), suggesting that mice immunized with CS-PLGA-rOmp22 vaccine showed decreased inflammatory response in lungs.

Survival rate, body weight changes and clinical score of mice post-challenge

Six mice challenged with *A. baumannii* were randomly selected for recording survival, body weight changes and clinical score every day for seven days. As shown in Fig. 8a-d, all mice in CS-PLGA-PBS and adjuvant treated groups died 72 h post-challenge. After the lethal dose of ATCC19606, CS-MDR-AB, CRAB and PDR-AB strains challenged, the 7-day survival rates of mice immunized with CS-PLGA-rOmp22 were 83.33%, 71.43%, 66.67% and 57.14%, respectively, which were significantly higher than those immunized with rOmp22 (60%, 60%, 50% and 42.86%, respectively).

After challenge, the body weight (Fig. 8e-h) and clinical symptom scores (Fig. 8i-l) of each group decreased to the lowest after two to three days post infection. Then, the symptoms of mice in the CS-PLGA-rOmp22 group and rOmp22 vaccinated group gradually improved. The body weight of mice returned to that before challenge, and the symptoms disappeared in seven days post challenge. These results demonstrated that mice immunized with CS-PLGA-rOmp22 nanovaccine were better protected from lethal dose of *A. baumannii* infection, compared with mice vaccinated with non-encapsulated rOmp22.

Discussion

Multidrug-resistant *A. baumannii* is a rapidly emerging pathogen causing infections with high mortality rates due to inadequate medical treatment[1, 2, 6]. New ways to prevent and treat such infections are of a critical medical need. Despite decades of effort in the development of *A. baumannii* vaccine, there is still no effective vaccine against this pathogen.

Recently, there is increasing interest in the development of vaccines which use only minimal components from pathogens. Such vaccines are based on recombinant proteins or even minimal fragments carrying immunological information from this protein, called peptide epitopes[24, 30]. Epitopes or antigenic

determinants are the minimal immunogenic part of any particular antigen, which are capable of inducing specific immune responses[24]. In this research, we chose *A. baumannii* Omp22, a highly conserved and highly immunogenic protein, as the candidate antigen. Bioinformatics techniques and immunological methods were used to predict and identify optimal T-cell and B-cell epitopes on Omp22 protein. Then, the identified dominant epitopes were connected in series by 6-aminocaproic acid and chemically synthesized to generate a novel multi-epitope peptide rOmp22, which preserved the antigenic epitopes and avoided toxic structure fragments of Omp22.

Peptide-based subunit vaccine holds great potential to be a safer and more efficient alternative to traditional vaccination strategies. However, nontoxic and effective delivery systems are needed to protect their respective immunogens from rapid degradation and to potentiate immunological responses[31]. A novel type of composite microspheres, CS-PLGA, was demonstrated to possess advantages of improving the stability of encapsulated proteins and increasing the subsequent release. In this view, we encapsulated a multi-epitope peptide rOmp22 in CS-PLGA NPs with the high encapsulation efficiency rate and slow release pattern. To the best of our knowledge, this study was the first to undertake the encapsulation of the multi-epitope peptide rOmp22 in CS-PLGA NPs, physical-structural characterization studies, and its immunogenicity and protection efficacy. This is also the first time that the CS-PLGA NPs delivery system has been used for epitopes vaccine research in bacteria.

The morphological characteristics of nanoparticles can affect the release of biomaterials from the nanoparticle. A leaky or porous structure enhances the release of biomaterials by diffusion, whereas a smooth surface reduces the burst release[32]. The physical and structural characteristics of CS-PLGA-rOmp22 revealed that it had a homogeneous morphology with a smooth spherical shape. CS-PLGA-rOmp22 was also uniform in particle size distribution and fairly dispersed without aggregation. The zeta potential value is one of the most important particle characterizations because it can affect both particle stability and particle adhesion[33]. As shown in Fig. 2e and 2f, CS-PLGA NPs exhibited positive charge, which could adsorb more peptides, increase encapsulation efficiency and improve stability.

The release pattern of a peptide in nanoparticles is important to the development of a nanovaccine, since it can influence the immune response and the immunization regimen, such as the peptide concentration and the frequency of immunization. In the present study, the CS-PLGA-rOmp22 release profiles were biphasic, characterized by a burst of peptide followed by a sustained release. The burst release at the first day could induce strong immune response. The sustained slow release of the peptide was an attractive property for a vaccine candidate as this might reduce the number of immunizations as well as enhancing the presentation of the peptide to APCs. Cell toxicity studies showed that CS-PLGA NPs was not toxic to cells at concentration as high as 800 µg/ml, thus indicating the safety of this delivery system as reported by several researchers[27, 28].

The present study showed that subcutaneous administration of CS-PLGA-rOmp22 nanovaccine induced systemic antibody responses. As shown in Fig. 4b-4d, high titers of antigen-specific antibody IgG were detected in the serum of mice immunized with CS-PLGA-rOmp22 after boosting twice. Meanwhile, CS-

PLGA-rOmp22 vaccination induced much higher levels of antigen-specific IFN- γ and IL-4 secretion in the spleen cell culture supernatants in CS-PLGA-rOmp22 and non-encapsulated rOmp22 groups compared to those in control mice. The CS-PLGA-rOmp22 vaccination induced much higher levels of antigen-specific IFN- γ production from splenocytes than rOmp22-immunized group ($P < 0.01$), whereas IL-4 secretion showed no difference between CS-PLGA-rOmp22 group and non-encapsulated rOmp22 group (Fig. 4e and 4f). These results indicated that humoral and cellular immune responses, especially Th1-type immune response had been induced and created a full protection.

The challenge experiments proved that the mice immunized with CS-PLGA-rOmp22 acquired potent protection against the infection of *A. baumannii* ATCC 19606 and three clinical *A. baumannii* strains. The bacterial load in the blood of the mice inoculated with CS-PLGA-rOmp22 after infection was much lower than that of non-encapsulated rOmp22 group and the control groups. Almost no pathological change was observed in the lung tissue of the mice immunized by CS-PLGA-rOmp22. These results indicated that the high titer of antigen-specific antibody contributed to the potent protection in the mice immunized with CS-PLGA-rOmp22.

Conclusions

In summary, this work reported a novel multi-epitope peptide nanovaccine against *A. baumannii*. CS-PLGA-rOmp22 NPs could elicit specific IgG antibody, Th1 cellular immunity and protection against acute lethal intratracheal *A. baumannii* challenge in BALB/c mice. Our results indicate this nanovaccine is a desirable candidate to prevent *A. baumannii* infection.

Materials And Methods

Materials

PLGA (lactide: glycolide = 50:50; MW = 30,000–60,000), polyvinyl alcohol (PVA; MW = 85 000-124 000, 99% hydrolyzed), HRP-labeled goat anti-mouse IgG, Freund's complete adjuvant and Freund's incomplete adjuvant were purchased from Sigma-Aldrich (St Louis, MO). The human lung adenocarcinoma epithelial cell line A549 was obtained from American Type Culture Collection (ATCC). RPMI-1640 medium, fetal bovine serum (FBS) and antibiotic-antimycotic were all purchased from Invitrogen (Carlsbad, CA). The Celltiter96 CCK8 Cell Proliferation Assay kit was purchased from KeyGEN BioTECH (Nanjing, China). Fc blocking antibody, Mouse I-Ab APC, PerCP-Cy5.5 anti-mouse CD11c, PE anti-mouse F4/80, FITC anti-mouse CD11b, PE anti-mouse CD3 and APC anti-mouse CD19 were all bought from BD-Biosciences (San Diego, CA). DNA ligase, DNA polymerase (Taq enzyme), restriction enzymes *Bam*HI and *Xho*I were purchased from American Thermo Company.

A. baumannii ATCC19606 strain was obtained from American Type Culture Collection (ATCC). Three clinical *A. baumannii* strains were collected from the Second Affiliated Hospital of Nanjing Medical University. All clinical *A. baumannii* strains were confirmed to be multi-drug resistant (MDR) strains by

drug sensitivity experiments (Additional file 1: Table S1) according to clinical and laboratory standards institute (CLSI) M100. The *E. coli* BL21 (DE3) and the plasmid pET28a (+) used in the study were purchased from Novagen company (Beijing, China) and kept in our laboratory. For all experiments, unless otherwise stated, bacteria were grown on Luria-Bertani (LB: 10 g/L tryptone, 5 g/L yeast extract, 10 g/L sodium chloride) agar plates or in LB broth at 37°C.

Animals

All animal experiments were performed using 6 to 8 weeks old female BALB/c mice purchased from Shanghai Super - B&K laboratory animal Corp. Ltd. (Shanghai, China). The mice were raised under specific pathogen-free (SPF) conditions at ambient temperature of 25 °C and provided with sterile food and water ad libitum. The animal experimental procedures were approved by the Ethics Committee of Animal Care and Welfare, Nanjing Medical University (Nanjing, China) (Approval Number: IACUC-1904044). All efforts were made to minimize animal suffering.

Expression and purification of Omp22

The gene encoding Omp22 was obtained through PCR amplification, using the genomic DNA of *A. baumannii* ATCC 17978 strain as the template. The primers were Omp22-F (5' CAA GGA TCC CTG GGC GGC GTT GAA TAT G 3') and Omp22-R (5' CAC AAG CTT TTA CTG TTT CGC GTA AAT G 3'). The PCR products was digested using the *Bam*HI and *Xho*I enzymes and ligated into the plasmid pET28a. The recombinant plasmid pET28a-omp22 was transfected into *E. coli* BL21 (DE3). The recombinant protein Omp22 was obtained through induced expression and purified. After eluted with elution buffer (1 M imidazole in binding buffer), the collected fractions were analyzed with SDS-PAGE.

Prediction and Identification of T-cell epitopes and B-cell epitopes

The physicochemical properties of *A. baumannii* Omp22 protein was analyzed and all possible dominant B-cell and T-cell epitopes were predicted using the immuno-informatics approach. The B-cell epitopes were predicted using bioinformatics software OptimumAntigen™ Design Tool (GenScript, China). Four candidate B-cell epitopes were predicted according to their secondary structure, surface accessibility, hydrophilicity, flexibility, and antigenic index. When predicting T-cell epitopes, The IEDB (Immune Epitope Database Analysis Resource) (<https://tools.iedb.org/mhci/>) was used and four T-cell epitopes were stored based on their scores[34, 35]. The predicted B-cell and T-cell epitope peptides were chemically synthesized by Jiangsu GenScript Biotechnology Co. Ltd.

Twelve female BALB/c mice (6 to 8 week) were randomly divided into two groups, Omp22 immunized group and phosphate-buffered saline (PBS) control group. Mice were subcutaneously injected with 100 µg recombinant Omp22 (1 µg/µl in PBS) or an equal volume of PBS for three times with two-week interval. In the Omp22 vaccine group, Freund's adjuvant was added to enhance the immune effect. One week after the last immunization, serum of each mouse was collected to detect B-cell epitope specific antigen by indirect ELISA. Splenocytes were isolated from vaccinated mice and adjusted to a

concentration of 1×10^6 cells/ml, and 200 μ l of the cell suspension was added to each well of a 96-well plate and stimulated with 20 μ g/ml of candidate T-cell epitopes. After incubated for 72 h, supernatants were collected and levels of gamma interferon (IFN- γ) were measured using mouse IFN- γ ELISA kits. Based on the above analysis, three B-cell epitopes (amino sequences: NIPLSQARAQSVKNY; YATLDKVAQTL and SVQLIMP) and two T-cell epitopes (amino sequences: VPSSRIDAQGY; TFDTNKSNIKP) from Omp22 were selected to design the multi-epitope protein.

Design and synthesis of rOmp22

Three optimal B-cell epitopes and two T-cell epitopes were connected in series by 6-aminocaproic acid. A multi-epitope peptide rOmp22 of 59aa, with a molecular weight of 6536.4 Da was chemically synthesized and identified by liquid chromatography and mass spectrometry. The synthesis and identification of rOmp22 was completed by Jiangsu GenScript Biotechnology Co. Ltd.

Preparation of nanoparticles

CS-PLGA NPs were prepared using a modified water/oil/water double emulsion evaporation technique[36]. Briefly, 2 mg of rOmp22 dissolved in PBS containing 0.003% sodium alginate was the inner water phase, 80 mg of PLGA (lactide: glycolide = 50:50; MW = 30000–60000) (Sigma-Aldrich) was dissolved in 2 ml dichloromethane (DCM) as the organic phase. 2% (w/V) polyvinyl alcohol (PVA; MW = 85000–124000, 99% hydrolyzed; Sigma-Aldrich) solution including 0.2% chitosan (MW = 50000; Sigma-Aldrich) was prepared as the external aqueous phase. The emulsion was stirred at room temperature overnight. The nanoparticles were obtained by ultracentrifugation, then washed three times with deionized water to remove excess polyvinyl alcohol and rOmp22, lyophilized to obtain CS-PLGA-rOmp22. An equivalent volume of PBS as used for rOmp22 was similarly encapsulated in CS-PLGA to obtain CS-PLGA-PBS to serve as a negative control. All lyophilized nanoparticles were stored at -80°C until used.

Encapsulation efficiency and peptide loading level

The encapsulation efficiency of rOmp22 in CS-PLGA was measured using HPLC (Agilent, USA) by quantitating rOmp22 in supernatant after ultracentrifugation. The rOmp22 encapsulation efficiency (EE) and the peptide loading capacity (LC) were calculated using the following formulas:

$$EE = (A-B) / A \times 100$$

$$LC = (A-B) / C \times 100$$

Where A is the total amount of rOmp22, B is the free amount of rOmp22, and C is the CS-PLGA-rOmp22 weight. These measurements were performed three times.

Particle size and zeta potential

The particle size, polydispersity index (PDI) and zeta potential were measured by particle size analyzer (Anton Paar, Graz, Austria). CS-PLGA-rOmp22 or CS-PLGA-PBS was suspended in filtered distilled water, sonicated, and placed in a cuvette to measure size and zeta potential. Each sample was measured three

times and reported as the mean of triplicates for size (diameter in nanometers) and zeta potential (millivolt). These experiments were conducted at least three times.

Transmission electron microscopy (TEM)

The morphology of CS-PLGA-rOmp22 and CS-PLGA-PBS were observed using high-resolution TEM (Hitachi, HT7700 Exalens). One drop of the complex was deposited on the copper grid (carbon-coated copper grid, 200 mesh). After adding phosphotungstic acid, the grids were dried for 10 min prior to TEM analysis.

In vitro peptide release

The release of the rOmp22 peptide from CS-PLGA was determined following the method of Bouissou et al[37]. Briefly, CS-PLGA-rOmp22 NPs were suspended in normal saline (NS). The suspensions were incubated at 37°C and at various time intervals (30 min, 1, 2, 4, 6, 12, 24, 48 and 72 h), supernatants were collected by centrifugation. The released peptide in the supernatants was measured using HPLC.

Cytotoxicity studies

The cytotoxicity of rOmp22, CS-PLGA-PBS and CS-PLGA-rOmp22 to human lung adenocarcinoma epithelial cell line A549 (ATCC) was detected by Cell Counting Kit-8 (CCK8) assay. In cell viability assay, A549 cell were seeded at 1×10^5 cells/well in 96-well plates in 100 µl of medium and incubated overnight at 37°C in a humidified atmosphere with 5% CO₂ to allow cell attachment. Then the cells were incubated with different concentrations of rOmp22 (1.25-80 µg/ml), CS-PLGA-PBS (12.5–800 µg/ml) or CS-PLGA-rOmp22 (12.5–800 µg/ml) for 6, 24 and 48 h. And then, 10 µl of CCK8 solution was added to the culture medium and incubated for an additional 2 h. Optical density (OD) values were measured at 450 nm wavelength using a microplate reader (Bio-Rad iMark).

Mouse immunization

BALB/c mice were randomly divided into four groups: CS-PLGA-rOmp22 treated group, CS-PLGA-PBS treated group, rOmp22-immunized group and an adjuvant-treated group. Mice in the CS-PLGA-rOmp22 group were immunized subcutaneously with 40 µg/200 µl of encapsulated rOmp22 in NS, and those in the CS-PLGA-PBS group were immunized with an equivalent weight of CS-PLGA-PBS NPs. Each mouse in the rOmp22 group was immunized with 40 µg non-encapsulated rOmp22 in 100 µl NS formulated with an equal volume of Freund's complete adjuvant (Sigma), and boosted with the same dose of rOmp22 in 100 µl NS formulated with Freund's incomplete adjuvant on day 14 and 28. In the adjuvant-treated group, each mouse was injected subcutaneously with 100 µl NS formulated with equal volume of Freund's complete adjuvant on day 0 or Freund's incomplete adjuvant on days 14 and 28. Six mice were randomly selected from each group for immunological assay. The other animals were used for challenge test two weeks after the third immunization.

Antibody titer measurement with ELISA

Multi-epitope peptide rOmp22 was first diluted to an optimal concentration (10 µg/ml) to coat a 96-well plate. The resulting solution was then added into each well (100 µl per well) and incubated for 12–18 h at 4°C. After washing five times with PBS plus 0.05% Tween 20 (PBST), 200 µl of 2% bovine serum albumin (BSA) was added to each well and incubated for 2 h at 37°C to block the unoccupied sites. After washing, serial dilutions of the pooled serum in each group ranging from 1:200 to 1:51200 were added to the wells in triplicate and incubated at 37°C for 2 h. Plates were washed 5 times as described above. And then 100 µl per well of Horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (diluted 1:10000) was added and the plates were incubated for 1 h. Plates were then washed 5 times and incubated with 100 µl per well of 3,3',5,5'-tetramethylbenzidine solution (TMB) as substrate until a desired absorbance was reached. The reaction was stopped by the addition of 100 µl of 2 M H₂SO₄. The optical density of the samples was measured at 450 nm using an ELISA plate reader. The endpoint titer was defined as the highest dilution of which the OD at 450 nm was at least 0.1 above that of the background well.

Splenocyte stimulation and cytokine secretion

Splenocytes were isolated as described previously from vaccinated mice seven days after the last immunization[10]. Splenocytes were adjusted to a concentration of 1×10^6 cells/ml, and 200 µl of the cell suspension was added to each well of a 96-well plate and either stimulated with rOmp22 (20 µg/ml) or left unstimulated. After incubated for 72 h, supernatants were collected and levels of gamma interferon (IFN-γ) and Interleukin 4 (IL-4) were detected using mouse IFN-γ and IL-4 ELISA kits.

Flow cytometry analysis

Single spleen cell suspensions and draining lymph nodes cell suspensions were obtained from mice one week after the last immunization. Cells (1×10^6 /ml) were blocked with Fc blocking antibody (BD Bioscience) in fluorescent-activated cell sorting (FACS) buffer (phosphate buffered saline, 1.0% fetal bovine serum) for 15 min at 4°C. The cells were washed and stained with fluorochrome-conjugated antibodies against lymphocyte surface receptors, APC-Mouse I-Ab, PerCP-Cy5.5-CD11c, PE-F4/80, FITC-CD11b, PE-CD3 and APC-CD19 (BD Biosciences) for 30 min at 4 °C. The cells were then washed and fixed with 2% paraformaldehyde solution for 20 min at 4 °C. Data were acquired on a BD FACS Canto II flow cytometer (BD Bioscience) with at least 1×10^5 events for each sample and analyzed using FlowJo software (Tree Star Inc, Ashland, OR, USA).

Establishment of pneumonia models

A. baumannii ATCC19606 strain and 3 clinical *A. baumannii* strains (CS-MDR-AB, CRAB and PDR-AB) were grown in LB broth to the late-logarithmic phase at 37°C/150 rpm. Cells were harvested by centrifugation at 4000 g for 10 min, washed and resuspended in PBS and mixed with porcine mucin (Sigma-Aldrich) to a final concentration of 5% mucin. Desired CFU/ml was obtained by appropriate dilutions and the final concentration was quantified by plating serial dilutions onto LB agar plates. The mice were anaesthetized with intraperitoneal (i. p.) injection of pentobarbital sodium, placed in a supine

position and their trachea were exposed surgically. Lethal doses of ATCC19606 (2×10^8 CFU) and three clinical *A. baumannii* strains, CS-MDR-AB (1×10^9 CFU), CRAB (5×10^8 CFU) or PDR-AB (5×10^8 CFU) in a total volume of 100 μ l was intra-tracheally to mice to induce acute pneumonia. The incised area was sealed with sterile surgical sutures. The mice were monitored for 7 days, body weight, clinical score and survival number from each group were recorded every day.

Bacterial load assessment in blood and lung

Blood samples were collected from six mice in each group at 24 h post-challenge. To determine the bacterial loads in blood, samples were serially diluted and plated on blood agar plates. After taking blood, the mice were killed, lungs were removed aseptically, weighed, and homogenized. Serial dilutions of tissue homogenates were plated onto blood agar plate. Bacterial CFUs were enumerated after 24 hours of incubation at 37°C.

Histopathological examination

Lungs were removed under aseptic conditions and fixed in 4% formalin. Histopathological examination of the section after embedding in paraffin was observed under microscope after staining with haematoxylin-eosin (HE). Lung injury was estimated by the percentage of the lesion area in the total lung area using an ImagePro macro.

Statistical analyses

Statistical analyses were performed using the Statistical Package of Social Sciences (SPSS, version 23.0; SPSS Inc., Chicago, IL) and Graphpad Prism (version 6.0; Graphpad software Inc., La Jolla, CA). All data were expressed as Mean $\pm$ SD. The one-way analysis of variance (ANOVA) was used for multiple comparisons, followed by Bonferroni's *post hoc* test. Survival data were compared using the log-rank test. A *P* value of < 0.05 was considered significant.

List Of Abbreviations

MDR: multi-drug resistant; PDR: pan-drug resistant; CS: chitosan; PLGA: poly lactic acid glycolic acid; NPs: nanoparticles; PBS: phosphate-buffered saline; HPLC: high performance liquid chromatography; TEM: transmission electron microscopy; PDI: polydispersity index; CRAB: carbapene resistant *Acinetobacter baumannii*; CFU: colony forming units; APC: antigen presenting cells.

Declarations

Authors' contributions

XRD and JPX contributed equally to this work. XRD performed the animal experiments, analysis and wrote the original draft. JPX conducted the fabrication and characterization experiments of the nanoparticles and revised the manuscript. MZJ, SQL, YZH, KLD, ZQL, SXC and ZYS performed the experiments, analysis, interpretation and statistic. LS, HMX and JY analyzed the data and revised the

manuscript. GZF conceived the project, obtained grant support and revised the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

The authors declare that all the relevant data supporting the findings of the study are available in the article and its Additional files, or from the corresponding author upon reasonable request.

Ethics approval and consent to participate

The care of the animals and all procedures used in these experiments were approved by the Ethics Committee of Animal Care and Welfare, Nanjing Medical University.

Consent for publication

Not applicable.

Competing interests

The authors declare no conflicts of interest in this work.

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Figures

Figure 1

Epitope prediction and design of multi-epitope peptide rOmp22. a Recombinant plasmid pET-28a-omp22. Lane M was the marker, lane 1 was the undigested pET-28a-omp22/BamH I , Hind III and lane 2 was the digested pET-28a-omp22. b SDS-PAGE of purified recombinant Omp22 protein. Lane M was protein Marker, lane 1, 2 and 3 were calibrator Bovine Serum Albumin (BSA) 1 μ g, 2 μ g and 4 μ g, respectively; lane 4 and 5 were His-omp22 protein 2 μ g and 4 μ g. c Immunization scheme of mice. BALB/c mice were subcutaneous (s.c) injected with Omp22 protein or PBS on day 0, 14 and 28. Blood and spleen were collected seven days after the last immunization. d Indirect ELISA test of candidate B-cell epitopes and Omp22 immunized mouse serum. Optimal B-cell epitopes were Omp22 B2: NIPLSQARAQSVK NY , Omp22 B3: YATLDKVAQTL and Omp22 B4: SVQLIMP. e IFN- γ levels in the splenocytes supernatant. Dominant T cell epitopes were Omp22 T1: TFDTNKSNIKP and Omp22 T3: VPSSRIDAQGY. f Design pattern of the multi-epitope peptide rOmp22, three optimal B-cell epitopes and two optimal T-cell epitopes were connected in series by 6-aminocaproic acid.

Figure 2

Physical-structural characterization of nanoparticles. TEM of CS-PLGA-PBS (a) and CS-PLGA-Omp22 (b), size distribution of CS-PLGA-PBS (c) and CS-PLGA-Omp22 (d), and zeta potential analyses of CS-PLGA-PBS (e) and CS-PLGA-Omp22 (f).

Figure 3

In vitro release of rOmp22 from CS-PLGA-rOmp22 NPs and cytotoxicity of CS-PLGA NPs to A549 cells. a In vitro release of rOmp22 from CS-PLGA-rOmp22 NPs. CS-PLGA-rOmp22 NPs were suspended in normal saline, incubated at 37°C at various time intervals (30 min, 1, 2, 4, 6, 12, 24, 48 and 72 h). b The cytotoxicity of rOmp22 to A549 cells. The A549 cells were incubated with rOmp22 at concentrations of 0, 5, 10, 20, 40, 80 µg/ml for 6, 24 and 48 h. c and d The cytotoxicity of CS-PLGA NPs to A549 cells. A549 cells were incubated with CS-PLGA-rOmp22 (c) or CS-PLGA-PBS (d) at concentrations of 0, 50, 100, 200, 400, 800 µg/ml for 6, 24 and 48 h. The cytotoxicity of rOmp22 and CS-PLGA NPs to A549 was detected by Cell Counting Kit-8 (CCK8) assay. Experiments were performed in triplicate.

Figure 4

Humoral and cellular immunity responses in BALB/c mice in one week after the last immunization. a BALB/c mice were immunized with CS-PLGA-rOmp22 and non-encapsulated rOmp22 on Day 0, 14 and 28. Blood, spleen and inguinal lymph nodes were collected one week after the last immunization. b rOmp22 specific IgG in serial dilutions from mice in one week after the last immunization. Data shown represent mean OD450 nm values (mean $\pm$ SD) for each group of six mice. c Endpoint titer is higher in the

CS-PLGA-rOmp22 group compared to non-encapsulated rOmp22 group. d rOmp22 specific IgG at 1:200 serum dilutions from mice in different groups. e The IL-4 concentration released from splenocytes supernatant of adjuvant control and immunized groups. f The IFN- γ concentration released from splenocytes supernatant of four different immunized groups.

Figure 5

Flow Cytometry analysis in the draining lymph node and splenocytes. a and b The scatterplot presents the percentage of CD 19+ B cells in the lymph node (a) and spleen (b) 7 days after the last immunization. c and d The scatterplot presents the percentage of CD 3+ T cells in the lymph node (c) and spleen (d) after booster vaccination. e and f The scatterplot presents the percentage of CD 11c+ dendritic cells in the lymph node (e) and spleen (f) 7 days post the last immunization. g and h The scatterplot presents the percentage of CD 11b+ CD 169+ cells in the lymph node (g) and spleen (h) 7 days after the last immunization. i and j Scatterplots present the major histocompatibility complex class II (MHC II) expression on CD 11c+ dendritic cells in the lymph node (i) and spleen (j). k and l Scatterplots present the major histocompatibility complex class II (MHC II) expression on CD 11b+ CD 169+ cells in the lymph node (k) and spleen (l). Statistics was performed using a one-way ANOVA with Tukey's post hoc correction (n=4). In all studies, *P < 0.05, **P < 0.01, ***P < 0.001. MFI, mean fluorescence intensity.

Figure 6

Bacterial loads in the blood and lung tissues of mice. **a** Immunization and *A. baumannii* challenge process. Two weeks after the last immunization, BALB/c mice were intratracheally infected with lethal dose of *A. baumannii* ATCC19606 (2×10^8 CFU/mouse) and three clinical *A. baumannii* strains, CS-MDR-AB (1×10^9 CFU/mouse), CRAB (5×10^8 CFU/mouse) and PDR-AB (5×10^8 CFU/mouse). **b** and **c** Bacterial loads in the blood of mice in 24 hours post-challenge. Blood samples were serially diluted and plated on LB agar plates followed by incubation at 37°C overnight. The number of colony forming units (CFU) was counted, and the $\log_{10}$ CFU/ml blood was calculated and compared. **d-g** Bacterial loads in lungs. Lungs were removed at 24 h after intra-tracheal challenge with *A. baumannii*. Bars indicate mean $\pm$ SD ($n=6$). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Figure 7

Lung histopathology. Lung specimens were taken from six mice challenged with *A. baumannii* in each group at 24 h post-challenge. Lung tissue sections were stained with haematoxylin-eosin and observed under a microscope ($\times 200$). a Lung tissue from different immunized groups suffered *A. baumannii* ATCC 19606 and three clinical *A. baumannii* strains (Scale bar, 50 μ m). b Lung tissue from unimmunized uninfected normal mouse showing normal histological characteristics (Scale bar, 50 μ m). c-f Semi-

quantitative analysis of the inflammation area in the lung tissue (n=6). Histograms show the mean percentage of lesion area within the total lung. Data are presented as the means± SD (n=6). * P < 0.05, ** P < 0.01, *** P < 0.001.

Figure 8

Survival rates, body weight changes and clinical score each day after *A. baumannii* challenge. Six mice challenged with *A. baumannii* were randomly selected for the observation and recording survival, body weight and clinical score everyday up to 7 days after challenge. a-d Survival rates, data are shown as the percentage survival at each time point. e-h Body weight of mice in each group post challenge. i-l Clinical score of mice post-challenge.

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