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Induction of humoral immune response against *Pseudomonas aeruginosa* flagellin$_{(1\text{-}161)}$ using gold nanoparticles as an adjuvant

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ABSTRACT

Flagellin of *Pseudomonas aeruginosa* is an important vaccine candidate. N-terminal domains are highly conserved in both type a and type b flagellins. The efficacy of gold nanoparticles (AuNPs) conjugated to N-terminal domains of *P. aeruginosa* flagellin (flagellin$_{(1\text{-}161)}$), as an immunogen in mice, has been assessed. The nanoparticles were conjugated to the recombinant protein through direct interaction of thiol molecules of the cysteines with AuNPs and formation of Au—S bond. Flagellin$_{(1\text{-}161)}$, AuNP-flagellin$_{(1\text{-}161)}$, and flagellin$_{(1\text{-}161)}$ emulsified in Freund's adjuvant (FA: complete/incomplete Freund's adjuvant formulation) were administered subcutaneously to BALB/c mice. Mice given AuNP-flagellin$_{(1\text{-}161)}$ elicited high titers of anti-flagellin$_{(1\text{-}161)}$ antibodies compared with non-immune group and/or mice which received flagellin$_{(1\text{-}161)}$ without adjuvant. In whole cell ELISA, these antibodies effectively recognized the native flagellin on the bacteria. Opsonophagocytosis assay demonstrated the functional activity and specificity of anti-flagellin$_{(1\text{-}161)}$ antibodies raised by AuNP-flagellin$_{(1\text{-}161)}$ against homologous strain. All of the results were comparable with those obtained by use of FA. Taken together, this is the first report of conjugation of AuNPs to flagellin and evaluating its immune response against *P. aeruginosa*.

1. Introduction

Q2 *Pseudomonas aeruginosa* is a gram-negative opportunistic bacterium that causes severe infections in immunocompromised individuals. *P. aeruginosa* is a major cause of nosocomial infections and is responsible for 10% of all hospital-acquired infections [1]. It has an array of chromosomal and plasmid-mediated antibiotic resistance factors, making *P. aeruginosa* infections severe, life-threatening and difficult to treat [2]. Accumulation of resistance after exposure to various antibiotics and cross-resistance between agents may result in multidrug-resistant (MDR) *P. aeruginosa* [1,3]. Emergence of these MDR strains indicates the importance of supplemental approaches to antibiotic therapy, such as immunoprophylaxis and/or immunotherapy. Essential to this end is the identification of highly immunogenic antigens suitable as vaccine candidates.

P. aeruginosa flagellum contributes to colonization and invasion during the early phase of infection in predisposed hosts and, therefore, has long been recognized as an important virulence factor in *Pseudomonas* infections [4]. Flagellum is made up of flagellin subunits and flagellins are potential vaccine candidates [5,6]. Flagellin's mode of action is mediated by binding to toll-like receptor 5 (TLR5), whose activation results in an enhanced transcription of immune response genes [7,8]. Studies have proposed that a short stretch of 10 amino acids (amino acids 88–97; LQRIRDLALQ) in the N-terminal region of *P. aeruginosa* flagellin might, at least in part, be an important domain in binding to TLR5 [9]. In addition to containing very important sequences for induction of immune responses, the N-terminal region of *P. aeruginosa* flagellin is highly conserved in both type a and type b flagellins. Therefore, it seems that antibodies raised against these protein domains may neutralize whole bacteria. Supporting this notion, Neville et al. demonstrated that antibodies raised against N-terminal domains (amino acids 1–156) of type-b flagellin afforded a remarkable improvement in survival in two lethal murine models of infection [10].

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As vaccine development is orientated toward less immunogenic "minimalist" compositions, formulations that boost immunogen effectiveness are increasingly needed [11]. Now, with only a few exceptions, aluminum-based compounds (alum salts) are the predominant human adjuvants in the majority of countries worldwide. In *P. aeruginosa* vaccine formulations used in clinical trials [12–14] aluminum hydroxide is the adjuvant used for systemic immunization. Unfortunately, the safety of aluminum adjuvants used in vaccinations is open to question. Growing evidence demonstrates direct and indirect neurotoxicity of aluminum salts [15,16]. Therefore, it is essential to seek other adjuvant formulations for vaccines.

Nanoparticles are currently receiving attention for biomedical applications [17]. Gold nanoparticles (AuNPs) show several features that make them well-suited for biomedical applications, including direct synthesis, stability, low toxicity and the ability to selectively incorporate with biomolecules such as proteins or peptides [18–21]. Importantly, growing evidence indicates that AuNPs as a carrier could induce macrophage activation [22], lymphocyte responses [23], and production of antibody against certain antigens [23–26]. Interestingly, it has been reported that AuNPs stimulate a stronger immune response than immunization using complete Freund's adjuvant (CFA) [25,27].

Based on these observations, we hypothesized that flagellin N-terminal domains (flagellin$_{(1\text{-}161)}$) would be highly immunogenic, and anti-flagellin$_{(1\text{-}161)}$ antibodies might recognize and neutralize whole bacteria. We also studied the capacity of AuNPs to induce antibody titer against flagellin in comparison with Freund's adjuvant (FA: complete/incomplete Freund's adjuvant formulation) and the capability of produced antibodies to recognize and neutralize *P. aeruginosa*.

2. Material and methods

2.1. Bacterial strains and animals

P. aeruginosa strain 8821M provided by Tarbiat Modares University Microbial Bank (Tehran, Iran). *Escherichia coli* strains DH5α and BL21 (DE3) were obtained from Invitrogen (USA, Carlsbad). Twenty female BALB/c mice (6–8 weeks old), for immunization and for opsonophagocytosis experiments, and one New Zealand white rabbit, for production of anti-flagellin$_{(1\text{-}161)}$ antibodies, were purchased from Pasture Institute (Karaj, Iran). All animal experiments were conducted in accordance with the protocols approved by the Tarbiat Modares University Animal Care and Use Committee (Tehran, Iran).

2.2. AuNPs preparation and characterization

Citrate-coated AuNPs were prepared by citrate reduction of tetrachloroauric acid ($HAuCl_4$) according to Turkevich's method [28] with some modifications. Briefly, 1 ml of $HAuCl_4$ (1 mg/ml) was added to 10 ml of trisodium citrate and the solution was heated to 70 °C and stirred for 1.5 h until the color turned to wine red. The shape, size and size distribution of AuNPs obtained were characterized by transmission electron microscopic analysis (Philips EM 208, Netherlands). The molarity and size of AuNPs were determined by UV–vis Spectroscopy (Biowave II, UK) [29].

2.3. Preparation of recombinant flagellin$_{(1\text{-}161)}$

After extraction of genomic DNA from *P. aeruginosa* 8821M, the coding sequence of *flagellin*$_{(1\text{-}161)}$ (Type a) (GenBank Accession No: JF523355) was PCR amplified using forward primer (5′ AATCCATGGCCTTGACCGTCAACACC 3′) containing *NcoI* (Sigma, USA) restriction site, and reverse primer (5′ AATAAGCTTGATGCCGACGCTGATG 3′) containing *HindIII* (Sigma, USA) restriction site. The purified PCR product was digested with *NcoI* and *HindIII* restriction enzymes and cloned into the same restriction sites of pET28a (Novagen, USA) expression vector to construct the pET28a-*flagellin*$_{(1\text{-}161)}$ vector. The recombinant vector transformed into *E. coli* DH5α competent cells. The cells were plated on LB agar (Merck, Germany) containing 25 μg kanamycin/ml (Sigma, USA) and incubated overnight at 37 °C. The following day, some kanamycin-resistant colonies were selected and analyzed by PCR, enzyme digestion and sequencing for the presence of *fagellin*$_{(1\text{-}161)}$. Afterwards, the recombinant pET28a-*flagellin*$_{(1\text{-}161)}$ vector transformed into competent *E. coli* BL21 (DE3) expression host. The transformed bacteria were plated on LB agar containing 25 μg kanamycin/ml and incubated overnight at 37 °C. The following day, a single resistant colony was cultured in 5 ml of LB medium supplemented with 25 μg kanamycin/ml and grown overnight at 37 °C. The recombinant bacteria cultivated overnight were diluted 1:100 with LB broth and subjected to further incubation. When OD_{600} reached 0.8, expression of protein was induced by adding IPTG (Sigma, USA) to the media at final concentration of 1 mM. The incubation was continued at 37 °C for a further 4 h. The expression of flagellin$_{(1\text{-}161)}$ protein from cells transformed with plasmid pET28a-*flagellin*$_{(1\text{-}161)}$ was analyzed by 12.5% SDS-PAGE and Western blot analysis, using anti-native flagellin and/or anti-flagellin$_{(1\text{-}161)}$ antisera. After expression of flagellin$_{(1\text{-}161)}$ on a large scale (1 L), the cells were harvested by centrifugation and subsequently resuspended in lysis/binding buffer (8 M urea, 20 mM sodium phosphate, 500 mM NaCl, pH 7.8) for 20 min at room temperature followed by centrifugation at 10,000 rpm for 20 min. The recombinant flagellin$_{(1\text{-}161)}$ protein was purified by Ni^{2+}-NTA resin column (Invitrogen, USA) under hybrid conditions (ProBond™ purification system instruction manual, Invitrogen) with some minor modifications. Briefly, the resin was equilibrated with denaturing binding buffer (8 M urea, 20 mM sodium phosphate, 500 mM NaCl, pH 7.8). The lysate was added to the column and mixed gently with the resin by rocking the column for 30 min. Afterwards, the column was washed using denaturing wash buffer, in which a linear urea gradient from 8 M to 1 M of wash buffer (20 mM sodium phosphate, 500 mM NaCl, pH 6.0) was applied. The washing was continued by applying native wash buffer (20 mM sodium phosphate, 500 mM NaCl, 20 mM imidazole, pH 8.0) to the column. Finally, the recombinant flagellin$_{(1\text{-}161)}$ was eluted in native elution buffer (20 mM sodium phosphate, 500 mM NaCl, 250 mM imidazole, pH 8.0). The purified protein was monitored by SDS-PAGE followed by coomassie brilliant blue G-250 staining (Serva, Germany). The purified flagellin$_{(1\text{-}161)}$ contained <50 endotoxin units per mg of the protein by LAL reagent kit (Charles River).

2.4. Preparation of AuNPs-flagellin$_{(1\text{-}161)}$

Flagellin$_{(1\text{-}161)}$ conjugated AuNPs were prepared by mixing AuNPs and flagellin$_{(1\text{-}161)}$ as described previously with slight modifications [30]. For example, 0.1 ml of 26.3 μmol flagellin$_{(1\text{-}161)}$ was added to 1.3 ml of AuNPs (1.94 nM) to achieve a ratio of ~1000 protein molecules/AuNP. The mixture was stirred for 1 h at 4 °C. Excess protein was removed from solution by centrifugation (17,000 × *g*, 30 min), supernatant was discarded and the protein-conjugated AuNPs were suspended in phosphate buffer pH 7.0. The conjugate stability was assessed by the absence of aggregation (color change from red to blue or gray) after adding 10% aqueous solution of NaCl. The conjugating efficiency was determined by quantifying the total amount of flagellin$_{(1\text{-}161)}$ used for conjugation and free flagellin$_{(1\text{-}161)}$ in the supernatant by measuring OD at 280 nm and Bradford protein assay method (Bio-Rad, USA). For both methods, the concentration of protein was determined by use of standard albumin curve. Finally, the conjugation was confirmed by UV–vis

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