

## The Potency of Aluminum Hydroxide Nanoparticles for Dengue Subunit Vaccine Adjuvant

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The potency of aluminum hydroxide as an adjuvant in vaccine development is considered to depend on its particle size. In previous studies, we have successfully prepared two size particle, micro, and nano, aluminum hydroxide gel (alum) adjuvants. The potency of those particles as a candidate of adjuvant is needed to be characterized. In this study, we formulated our adjuvants with purified DENV3 pre Membrane Envelope (prM-E) recombinant protein and evaluated the induction of nitric oxide level in mouse macrophage RAW 264.7 cells. We prepared the alum adjuvant by precipitation-homogenization methods with an agitation rate at 11,000xg. Secreted prM-E recombinant protein was collected from *Pichia pastoris* X-33 fermentation which produced using bioreactor. Recombinant protein purification was carried out by anion exchange chromatography followed with size exclusion chromatography. The purified prM-E recombinant protein was observed as a single band around 70 kDa with a concentration of 105 µg mL<sup>-1</sup>. Complex nanoparticles alum with prM-E protein significantly (p<0.05) induced the nitric oxide level. Further analysis should be conducted in order to discover the detail molecular mechanism of nanoparticle alum adjuvant, recombinant protein, and cellular immune response.

Key words: adjuvant, dengue, nitric oxide, prM-E

Potensi aluminum hidroksida (alum) sebagai adjuvan vaksin sangat bergantung dari ukuran partikelnya. Pada penelitian sebelumnya, kami telah berhasil mengembangkan dua jenis ukuran partikel adjuvan dari alum, yaitu mikron dan nano. Pada penelitian ini, telah dilakukan karakterisasi dari kedua jenis partikel alum yang digunakan dalam pengembangan kandidat adjuvan vaksin demam berdarah. Partikel adjuvan diformulasi dengan protein rekombinan prM-E DENV3 dan dievaluasi potensinya dalam menginduksi senyawa *nitric oxide* (NO) pada galur sel mencit RAW 264.7. Partikel alum disiapkan dengan metode presipitasi-homogenisasi melalui proses agitasi pada kecepatan 11.000xg. Protein rekombinan prM-E diproduksi dari sel ragi *Pichia pastoris* X-33 melalui proses fermentasi menggunakan biorektor. Purifikasi protein rekombinan dilakukan menggunakan metode *ion exchange* yang dilanjutkan dengan *size exclusion chromatography*. Protein rekombinan yang berhasil dipurifikasi divisualisasi sebagai pita protein tunggal yang berukuran sekitar 70 kDa dengan konsentrasi sekitar 105 µg mL<sup>-1</sup>. Hasil dari penelitian pendahuluan ini menunjukkan bahwa kompleks alum adjuvan nanopartikel dengan protein rekombinan prM-E secara signifikan (p<0,05) menginduksi kadar NO dibandingkan dengan kompleks alum lainnya. Penelitian lanjutan perlu dilakukan untuk mendapat informasi yang lebih detil mengenai mekanisme molekular antara alum adjuvan nanopartikel, protein rekombinan dan respon imun seluler.

Kata kunci: adjuvan, dengue, *nitric oxide*, prM-E

Vaccine adjuvant promotes the production of long-lasting, efficient and specific immune responses. In the same time, the adjuvant improves the protective effect of vaccines due to a higher antibody yield and the persistence of antibodies, as well as functional T cells at high levels. Freund's adjuvants are the most common adjuvant used in experimental animals. It can enhance strong antigen-specific immune responses but at the same time, it causes strong inflammation and necrosis at the injection site, which prevents its use in vaccine development. Aluminum hydroxide adjuvants (alum)

are often used in clinical trials and have a reputation of safety and the facilitation of long-lasting antibody responses (Sun *et al.* 2014). Alum has been widely used as human vaccine adjuvants for almost a century. It is known that their immunostimulation effect is associated with the induction of Th2 responses (Ulanova *et al.* 2001). It has also been demonstrated that alum enhances antigen uptake by the antigen-presenting cells in vitro (Mannhalter *et al.* 1985).

Nitric oxide (NO) has been known as one of the most important components in the immune system. It is involved in the pathogenesis and control of many infectious diseases, cancers, autoimmune and chronic degenerative diseases (Bogdan 2001). Initially, NO

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was described as a physiological mediator of endothelial cell relaxation plays an important role in hypotension. Several cells of the innate immune system such as macrophages, neutrophils, and natural killer (NK) cells use pattern recognition receptors to recognize molecular patterns associated with pathogens. Activation of macrophages inhibits pathogen replication by releasing a variety of effector molecules, including NO. Nitric oxide is important as a toxic defense molecule against infectious organisms. It also regulates the functional activity, growth, and death of many immune and inflammatory cell types including macrophages, T lymphocytes, antigen-presenting cells, mast cells, neutrophils and NK cells (Tripathi 2007). A previous report showed that chicken spleen macrophage produced NO after treated by liposomes adjuvant. The production of NO by activated macrophages is an important index of immune stimulatory activity by an adjuvant (Lin *et al.* 2011).

Dengue virus (DENV) is a major cause of morbidity and mortality in tropical and subtropical regions, causing hundreds of millions of infections each year around the globe. Infections range from asymptomatic to a self-limited febrile illness, dengue fever (DF), to the life-threatening dengue hemorrhagic fever/dengue shock syndrome (DHF/DSS) (Guo *et al.* 2017). Neither antiviral nor vaccine is available in the market to control dengue infection. DENV vaccine development is challenging due to the existence of four serotypes of the virus (DENV1–4), which a vaccine must protect against all serotypes (Chokephaibulkit *et al.* 2013). DENV is a member of the Flaviviridae family and is grouped within the flavivirus genus. The viral genome consists of a positive-sense RNA of ~11 kb which encodes 3 structural proteins (capsid, pre-membrane and envelope) that form the components of the virion, and 7 non-structural proteins (NS1, NS2A/B, NS3, NS4A/B, NS5) involved in viral RNA replication (Perera *et al.* 2008). Several groups have been exploited the use of pre-membrane and envelope protein as the target candidate in the development of a subunit dengue vaccine (Kaufman *et al.* 1987; Robert Putnak *et al.* 2005). These proteins are immunogenic and confer some degree of protection against live virus challenge in animal models. The previous study showed that the formulation between these proteins with alum adjuvant could elicit virus-neutralizing antibodies in mice and rhesus macaques and confer at least partial protection against virus challenge (Putnak, Barvir *et al.* 1996; Putnak, Cassidy *et al.* 1996).

In the present study, we aimed to evaluate the induction of nitric oxide level in mouse macrophage RAW 264.7 cells treated with several formulations of complex alum adjuvant with DENV3 recombinant prM-E protein. The results of this preliminary study will improve our understanding of the best adjuvant formulation for dengue vaccine development.

## MATERIALS AND METHODS

**Aluminum Hydroxide Preparation.** Aluminum hydroxide was prepared by the precipitation-homogenization method as described in the previous report (Mardiyati 2017). An equal volume of 3.60 mg ml<sup>-1</sup> AlCl<sub>3</sub>.6H<sub>2</sub>O was mixed with 0.04 M sodium hydroxide dropwise and stirred at 20xg. A small volume of 0.01 M NaOH was added to adjust the pH to 6.8. After 24 hours of decantation, suspension of aluminum hydroxide microparticles was separated and washed twice with phosphate buffer pH 6.8 in order to remove sodium chloride in the suspension. Aluminum hydroxide was precipitated until aqueous suspension of aluminum hydroxide gel adjuvant (alum) was formed. Aluminum hydroxide nanoparticles were synthesized by downsizing (to break down particle size) it with high shear homogenization at 11,000xg for 1 hour. Sodium polyphosphate 0.40% was added before downsizing to make aluminum hydroxide nanoparticles more stable. The particles size distribution was determined using a Particle Size Analyzer (Horiba LA95A).

**Mice Macrophage RAW 264.7 Cells.** The RAW 264.7 mouse macrophage cell line (a kind gift from Dr. Peik, KRIBB Korea) was maintained in RPMI complete media supplemented with 10% Fetal Bovine Serum (FBS) and 1% penicillin-streptomycin in the presence of 5% CO<sub>2</sub>. Cells were passaged every three days in order to maintain the condition of the cells.

**Production of DENV3 prM-E Recombinant Protein.** Recombinant DENV3 prM-E protein was constructed from DENV3 clinical isolates which collected during cross-sectional study which was conducted on 2009 in Jakarta (Lestari *et al.* 2017). The prM-E gene was cloned and expressed in *Pichia pastoris* X-33 (unpublish data). High-cell-density cultivations of *Pichia pastoris* secreting DENV3 prM-E recombinant protein were carried out in the bioreactor (BioFlow120, Eppendorf) with slight modification (Gurramkonda *et al.* 2010). Precultures for high-cell density bioreactor cultivations were prepared in 100 ml for overnight at 30°C with agitation

Table 1 Physicochemical properties of aluminum hydroxide

No	Sample	Average particle size ( $\mu\text{m}$ )
1	Alhydrogel <sup>®</sup>	$0.913 \pm 0.8316$
2	Microparticle adjuvant	$5.671 \pm 0.3261$
3	Nanoparticle adjuvant	$0.243 \pm 0.0008$

around 25xg. The overnight starting culture was transferred to the bioreactor containing 2 liters Ursula Rinas medium. The temperature was maintained at 30°C and pH at pH 5.5 with 12.5% (v/v)  $\text{NH}_4\text{OH}$ . Aeration rate was maintained at 4  $\text{L min}^{-1}$  throughout the process. The stirrer speed was controlled between 30 to 100xg aiming at dissolved oxygen (DO) concentration of 30% air saturation. In this study, we induced the secretion of prM-E protein by applying 2.5% methanol induction for 48 hours. The supernatant was collected by centrifugation followed by buffer exchanged with 20 mM Tris-Cl pH 8 prior purification with anion chromatography by using DEAE resin from GE (UK). Buffer 20 mM Tris-Cl pH 8 with 1M NaCl was used as elution buffer. Subsequently, the size exclusion chromatography was performed using resin Sephacryl HR200 (GE) with PBS buffer in order to purified single band of prM-E recombinant protein. All purified protein was confirmed by using SDS PAGE analysis and the concentration of the protein was determined by BCA protein assay kit (Thermo Scientific).

**Cell Viability Assay.** To evaluate the cytotoxicity of the alum adjuvant to the cells, the 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium-bromide (MTT) assay was performed as described previously (Mosmann 1983). The mouse macrophage RAW 264.7 cells were seeded on 96-well plate at  $1 \times 10^5$  cells  $\text{ml}^{-1}$ . After 16-20 hours, cells were incubated in triplicate for 24 hours with a various concentration of alum ranging from 0.01% to 1.0% in 2% FBS-RPMI. After incubation, culture media with alum was removed from the cells and directly incubated with 50  $\mu\text{l}$  of MTT solution (PBS containing 0.5  $\text{mg ml}^{-1}$  MTT) for 4 hours at 37°C. The MTT solution was then replaced with 100  $\mu\text{l}$  of 10% SDS solution followed by incubation in the dark room for 22 hours. After gentle shaking for 10 min at room temperature, the absorbance at 570 nm was measured using a Universal Microplate Reader ELx800 (BIO-TEK Instruments, Inc.). The cell viability percentage for each adjuvant concentration was calculated by using GraphPad Prism 5 software.

**Nitric Oxide (NO) Assay.** The production of NO was determined by measuring the quantity of nitrite in the supernatant by the Griess reaction (Giustarini *et al.* 2008). The RAW 264.7 cells were seeded on 96-well plate at  $1 \times 10^5$  cells  $\text{ml}^{-1}$ . After 2 hours, cells were incubated in triplicate for 24 hours with various concentrations of alum ranging from 0.001% to 1.0% in 2% FBS-RPMI with and without 10  $\mu\text{g}$  purified prM-E recombinant protein. Supernatant from the experimental cell culture was removed to another well plate in triplicate and dispense of Griess solution with ratio 1:1 to all experimental samples followed by incubation in the darkroom for 10-20 minutes. The absorbance at 540 nm was measured using a Universal Microplate Reader ELx800 (BIO-TEK Instruments, Inc.) to determine the amount of NO in the samples.

**Statistical Analysis.** The results were analyzed using the one way ANOVA with Tukey's Multiple Comparison Test. Differences were considered significant (\*) at  $p$  value < 0.05.

## RESULTS

**Aluminum Hydroxide Particle Size.** The particle size of the alum adjuvant was determined by using PSA as shown in Table 1. The size of commercial Alhydrogel in this study was around  $0.913 \pm 0.8316$   $\mu\text{m}$ . High shear homogenization at 11,000xg for 1 hour could reduce the size of alum adjuvant particle from  $5.671 \pm 0.3261$   $\mu\text{m}$  to  $0.243 \pm 0.0008$   $\mu\text{m}$ .

**Production of Recombinant DENV3 prM-E Protein.** We performed the production of *Pichia pastoris* expressing prM-E recombinant protein using bioreactor system in order to maximize the biomass cells and secreted protein. Several major bands (200 kDa, 180 kDa, 100 kDa, 70 kDa, 10 kDa) appeared from the supernatant before purification step. The collected supernatant was subjected for anion exchange and size exclusion chromatography purification steps. A single band of prM-E protein around 70 kDa appeared with a concentration of 105  $\mu\text{g ml}^{-1}$  after purification step (Fig 1).

**Cell Viability of the Aluminum Hydroxide.** The

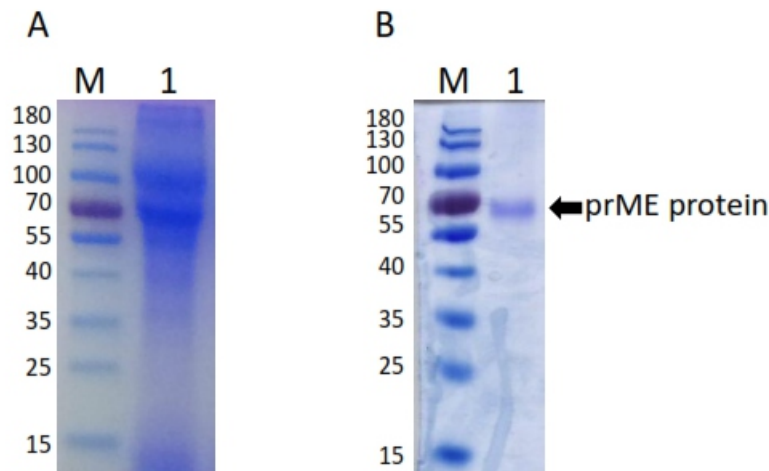


Fig 1 Recombinant prM-E DENV3 purification. (A) Total secreted protein in supernatant before purification. In line 1, several bands appeared mainly at 200 kDa, 180 kDa, 100 kDa, 70 kDa and 40 kDa. (B) Purified prM-E recombinant protein after anion exchange followed by size exclusion chromatography. In line 1, prM-E recombinant protein appeared clearly with the correct size around 70 kDa.

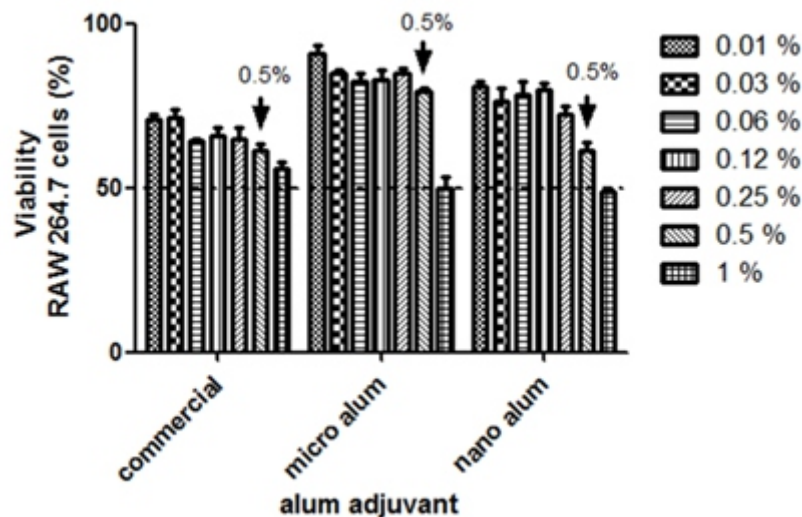


Fig 2 RAW 264.7 cell viability. Mouse macrophage RAW 264.7 cells were seeded on 96-well plate at  $1 \times 10^5$  cells  $\text{ml}^{-1}$ . After 16-20 hours, cells were incubated in triplicate for 24 hours with various concentrations of alum ranging from 0.01% to 1.0% in 2% FBS-RPMI. At concentration 0.5%, more than 60% of cells were survived after the treatment with alum adjuvant.

cell viability was evaluated by MTT assay. The viability of RAW 264.7 cells was decreased in a dose-dependent manner for all particle size of alum adjuvant, including for the commercial alhydrogel<sup>®</sup> adjuvant. Almost 50% of mouse macrophage RAW 264.7 cells were killed after treatment with 1% of alum adjuvant. However, at a concentration of 0.5%, more than 60% of cells were survived and we used this alum concentration for further analysis (Fig 2).

**Induction of Nitric Oxide.** No nitric oxide was induced in mouse macrophage RAW 264.7 cells after treated with all alum adjuvant alone (Fig 3). Interestingly, recombinant prM-E protein alone could induce nitric oxide compared to negative control

(media or alum only). Moreover, nitric oxide was released significantly ( $p < 0.05$ ) from mouse macrophage RAW 264.7 cells after treated with nanoparticle alum adjuvant which was formulated with prM-E recombinant protein (Fig 3).

## DISCUSSION

There is limited study reported the effect of alum particles size on the subsequent immunogenicity of the adjuvanted vaccine. In these studies, we evaluated the cytotoxicity and nitric oxide level induced by complex alum adjuvant with DENV3 recombinant prM-E protein in mouse macrophages RAW 264.7 cells. Two



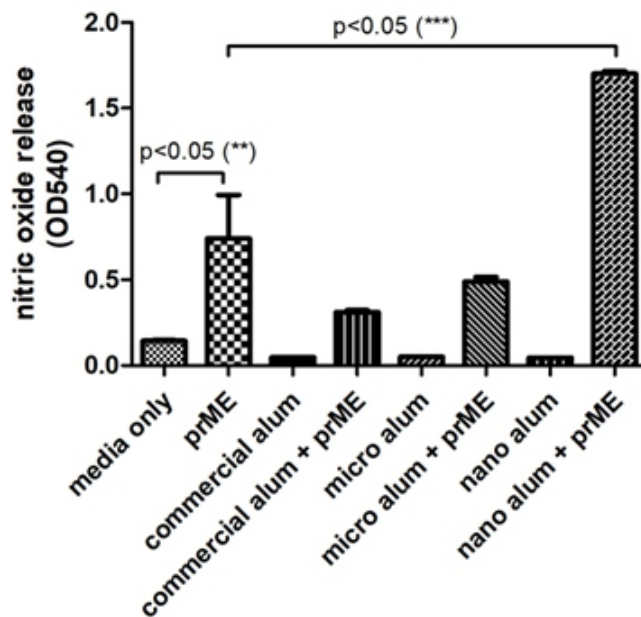


Fig 3 Induction of nitric oxide. (A) Mouse macrophage RAW 264.7 cells were treated with alum adjuvant only or with alum prM-E complexes. Alum alone could not induce immune response however prM-E without adjuvant could induce NO. Nanoparticle alum complexes induced nitric oxide significantly ( $p < 0.05$ ) compared to other groups.

different sizes of alum particle, nano and micro, were successfully prepared in the previous study (Mardiyati 2017). The toxicity of our alum particles was evaluated in this study. Viability of mouse macrophages RAW 264.7 cells showed slightly better when treated with our alum particles compared to the commercial alhydrogel<sup>®</sup> adjuvant (Fig 2). The previous study reported the mechanisms of action of alum of minor toxicity in some subjects by inducing cell death and inflammasome and developed persistent lumps and granulomas at the injection site (Petrovsky 2015).

Nitric oxide has been known as one of a key player in immune response. To evaluate the possibility of our alum adjuvant to induce nitric oxide, we treated the mouse macrophage RAW 264.7 cells with of micron and nano alum adjuvant together with commercial alhydrogel<sup>®</sup> adjuvant as a control.

Similar to commercial alhydrogel<sup>®</sup> adjuvant, our results showed neither micron nor nanoparticle alum alone could not induce nitric oxide (Fig 3). Contrary, a previous study showed that alum adjuvant alone could induce several cytokines and chemokines from macrophage cells of injected mice (McKee *et al.* 2009). The different immune response mechanism of alum adjuvant in vitro and in vivo system could be used to explain those phenomena.

Immunization of DENV prM-E protein demonstrating the immunogenicity and protective efficacy against DENV infection in small animals.

However, the prM-E protein that used on most DENV vaccine study was formulated with adjuvant (Mani *et al.* 2013; Urakami *et al.* 2017). Interestingly, the result from these studies showed that prM-E alone is enough to induce NO level compared to control alum without prM-E protein (Fig 3). Development of DENV vaccine by stimulating immune responses against the DENV pre-membrane and envelope (prM-E) protein have been reported elsewhere (Guirakhoo *et al.* 2000; Liu *et al.* 2016). The envelope protein of DENV is responsible for a wide range of biological activities, stimulates host immunity responses by inducing protective and neutralizing antibodies, including binding to host cell receptors, fusion and entry into host cells. Therefore, the dengue prM-E protein is an important antigen for vaccine development (Fahimi *et al.* 2018).

The nanoparticle alum prM-E complexes significantly induced immune response compared to other formulation (Fig 3). A previous study showed that 3  $\mu\text{m}$  aluminum phosphate particles had better uptake than 17  $\mu\text{m}$  aluminum hydroxide particles. Alum aggregates below 10  $\mu\text{m}$  are needed for efficient uptake of vaccine by APCs (Morefield *et al.* 2005). The size of nanoparticle alum in this study is less than 1  $\mu\text{m}$  (243 nm) and theoretically makes the uptake of prM-E protein by the cells more efficient compared to other formulations.

Alum greatly enhances priming of endogenous

CD4 and CD8 T cells independently of mast cells, macrophages and of eosinophils. However, activation of type 2 innate response orchestrated by macrophages and mast cells *in vivo* are not required for alum's adjuvant effects on endogenous T and B cell responses (McKee *et al.* 2009). In the field of drug delivery, nanoparticles have been shown to have a number of advantages over larger micron-sized particles. These advantages include increased intracellular uptake, improved bioavailability for poorly water-soluble drugs and improved pharmacokinetics, particularly in regards to tumor targeting where nanoparticles can prolong circulation of the drug, avoiding the reticuloendothelial system leading to a longer circulation time and improved efficiency of delivery to the tumor (Shah *et al.* 2014). To the best of our knowledge, there is no study has been reported regarding the evaluation of inducible nitric oxide by nanoparticle alum adjuvant formulated with DENV recombinant prM-E protein using *in vitro* system. Further analysis should be carried out in order to discover the molecular mechanism interaction of specific particle size of alum adjuvant, recombinant protein, and cellular immune response. This kind of studies would enhance our knowledge about the detail mechanism and function of alum adjuvant in vaccine development.

## ACKNOWLEDGMENT

This research was supported by Insinas Research Grant from the Ministry of Science, Technology and Higher Education of the Republic of Indonesia year 2018.

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